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Current evidence for the hepatoprotective activities of the medicinal mushroom *Antrodia cinnamomea*

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Abstract

Antrodia cinnamomea (AC) is an endemic mushroom species of Taiwan, and has been demonstrated to possess diverse biological and pharmacological activities, such as anti-hypertension, anti-hyperlipidemia, anti-inflammation, anti-oxidation, anti-tumor, and immunomodulation. This review focuses on the inhibitory effects of AC on hepatitis, hepatocarcinoma, and alcohol-induced liver diseases (*e.g.*, fatty liver, fibrosis). The relevant biochemical and molecular mechanisms are addressed. Overall, this review summarizes the hepatoprotective activities *in vitro* and *in vivo*. However, there is no doubt that human and clinical trials are still limited, and further studies are required for the development of AC-related products.

Introduction

Antrodia cinnamomea (AC) (*niu-chang-chih* in Chinese) is a medicinal mushroom (Figure 1) endemic to Taiwan. It has been well-known for its medicinal properties, particularly with regard to liver complaints [1], since its first identification as a new species by Zang and Su in 1990 [2]. Among its diverse pharmacological activities, the evidence for hepatic protection is the most recognized and the strongest. Studies have shown that AC can inhibit hepatic tumor growth and retard the progression of hepatitis [3,4]. Although AC is currently used as a food supplement, the Food and Drug Administration (FDA) has not approved any AC extracts or purified compounds for clinical applications. Some AC products are claimed to protect the liver against food and drug intoxication, especially alcohol-induced liver damage, maintain hepatic homeostasis, or both. This article reviews the current evidence for the hepatoprotective properties (such as effects on hepatitis, cirrhosis, liver cancers, and alcoholic damage) of AC extracts and active compounds.

Effects on hepatitis
General background of hepatitis

Hepatitis is one of the top ten causes of death worldwide (more than one million deaths per year), and is mainly classified into hepatitis A, B, and C, involving different types of viruses, epigenetic and genetic alterations, pathological progressions, and clinical treatments [5]. Hepatitis A is an acute infectious liver disease caused by hepatitis A virus (HAV). It is transmitted by a fecal-to-oral route, and mainly occurs as outbreaks from fecal contamination of food or water sources or direct contact with carriers. It has no clinical signs or symptoms in most infected people, and most cases recover within 1–2 months [6]. Hepatitis A causes the least problems among the various types of hepatitis, and its incidence rate has been diminishing in recent years, probably through improved hygiene [6]. Hepatitis B and C are acute and chronic infections caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively, and are more serious. HBV and HCV belong to the Hepadnaviridae and Flaviviridae, respectively. HBV is a DNA virus that can integrate into the host genome after infection, while HCV is an RNA virus that has no such ability [6]. According to a worldwide epidemiological evaluation, at least 400 and 170 million people are chronically infected with HBV and HCV, respectively [7,8]. In fact, these viruses persist in the liver in about 80% of infected people [9], and such chronic viral infections may be

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Figure 1 Morphological appearance of *Antrodia cinnamomea*.

associated with chronic inflammation that leads to scarring of the liver tissues and cirrhosis [10]. Upon infection, HBV and HCV interact with host hepatocytes, impair cell metabolism (*e.g.*, induce cell death) [11], modulate signaling pathways of protein synthesis (*e.g.*, produce viral proteins and enzymes) [12,13] and immunity (*e.g.*, synthesize and place viral antigens on host cell surfaces) [14], and develop immortalization (*e.g.*, modulate surface markers). Ultimately, 1–5% of cirrhotic livers develop hepatocellular carcinoma (HCC) [15].

Anti-hepatitis activity
Inhibition of viral proteases, which are crucial for viral replication, can interrupt viral protein maturation. Antrodins (A to E) and their metabolic analogues selectively inhibited HCV NS3/4A protease activity [3]. Among these, antrodin A (the major *in vivo* metabolite of antrodin C) showed the highest potency with an IC_{50} of 0.9 μg/mL, while antrodin B showed lower potency with an IC_{50} of > 100 μg/mL. Moreover, polysaccharides isolated from fruiting bodies and cultured mycelia of AC inhibited HBV replication activity [16]. After an HBV-producing cell line (MS-G2) was treated with the AC-extracted polysaccharides, anti-hepatitis B surface antigen (HBsAg) and anti-hepatitis B e antigen (HBeAg) were reduced [16]. Moreover, the AC-extracted polysaccharides (50 μg/mL) were found to be more effective than interferon-α (IFN-α) (1000 U/mL) for HBsAg and HBeAg inhibition [16]. Huang et al. [17] showed that 2,2′,5,5′-tetramethoxy-3,4,3′,4′-bis(methlylene-dioxy)-6,6′-dimethylbiphenyl (50 μM) could suppress the HBsAg and HBeAg levels in a wild-type HBV-producing cell line (ES2) within a non-toxic range and in a dose-dependent manner. These findings indicate that

AC can effectively attenuate hepatitis virus-induced damage by inhibiting essential viral enzyme activities and antigen production.

Effects on HCC
Pathogenesis of HCC
Liver cancer comprises diverse hepatic neoplasms, including HCC, cholangiocarcinoma, hepatoblastoma, and hemangiosarcoma [18,19]. Among these, HCC is the primary malignant hepatic cancer worldwide, the fifth most common cancer, and the third leading cause of cancer death around the world [20-22]. The incidence and mortality of HCC are both increasing [23-26]. HCC arises in the context of chronic viral hepatitis (*e.g.*, HBV, HCV, and HDV infection), cirrhosis, alcoholism, toxin or drug poisoning (*e.g.*, aflatoxins), and metabolic disorders (*e.g.*, diabetes, non-alcoholic fatty liver disease, and hereditary diseases) [27]. Recently, geographical differences in HCC incidence have been related to the changing distribution and natural history of HBV and HCV infections [28]. The World Health Organization (WHO) reported that HBV is a human carcinogen, and that an estimated 300–400 million individuals are chronically infected with HBV worldwide [29]. Moreover, a large number of carriers fail to eliminate the virus, and progress to chronic viral infection. Continuous hepatic inflammation can lead to chronic hepatitis and cirrhosis. The incidence of HCC in chronically HBV-infected or cirrhotic individuals is 100- and 1000-fold higher, respectively, than in uninfected individuals [27,30]. For toxin-induced HCC, ingestion of aflatoxin-B1 (a fungal toxin classified as a type I carcinogen by the International Agency for Research on Cancer (IARC) and produced by the molds *Aspergillus flavus* and

Aspergillus parasiticus found in contaminated food) increased the risk of HCC development [31,32]. Aflatoxin-B1 also induced mutations in codon 249 of the *p53* tumor suppressor gene and the *RAS* oncogene [33-36]. Alcohol, which is also classified as a type I carcinogen, is another critical HCC risk factor [31,32]. Alcohol activates monocytes and induces pro-inflammatory cytokine production, and the subsequent increase in endotoxins activates Kupffer cells to release chemokines and cytokines (*e.g.*, tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6)). The resulting oxidative stress activates stellate cells to synthesize large amounts of collagen, leading to fibrosis, cirrhosis, and ultimately HCC [37-39]. Apart from these etiological agents, inflammation, oxidative stress, genetic alternations, activation of oncogenes, inactivation of tumor suppressor genes, genomic instability, and overexpression of growth and angiogenic factors are related to the pathogenesis of HCC [31,40].

Angiogenesis plays an important role in the pathogenesis (*i.e.*, tumor growth, invasion, and metastasis) of all types of solid tumors, including HCC [41]. As a hypervascular tumor, HCC develops and progresses from a small, avascular, and well-differentiated HCC to a large, highly vascular, and poorly differentiated HCC within a short period [42]. The expanded endothelium provides a channel for HCC cells to enter the blood circulation system and metastasize to different tissues. During tumor angiogenesis, angiogenic factors (*e.g.*, vascular endothelial cell growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiogenin, or angiopoietins) and their receptors are produced by tumor cells and endothelial cells (ECs) [43,44]. Through VEGF signaling, the most common and potent angiogenic signaling pathway, ECs express VEGF receptors (VEGFR-1 and -2), receive signals from tumor-generated VEGF, proliferate, migrate toward tumor tissues, and form functional neovessels [43,44]. Moreover, these angiogenic factors induce gene transcriptional, biochemical, and enzymatic activities, such as extracellular digestion of extracellular matrix by matrix metalloproteinases (MMPs), cell cycle progression, and production of bioactive molecules (*e.g.*, angiogenic factors, cytokines, and chemokines) [43,44]. As a result, both tumor cells and ECs are activated in a feedback mechanism, thereby facilitating tumorigenesis [45].

Anti-hepatocarcinoma activity

Various types of AC extracts and purified components (*e.g.*, antcin A, B, and C, antroquinonol, methyl antcinate A (MAA), methyl antcinate B (MAB) ethylacetate, and methanolic, ethanolic and fermented extracts) exhibit cytotoxicity through apoptosis in various cancers, especially hepatic carcinoma. Apoptosis is processed sequentially through the activation of death receptors (*e.g.*, Fas receptor) and downstream caspases (*e.g.*, caspase-3, -8,

and -9), and mitochondrial function disruption. MAA (30.2–78 μM) and antcins A and C (30.2–286.4 μM) stimulate a series of apoptotic pathway cascades, including caspase-2, -3, and -9 activation, Bax, Bak, and PUMA protein expression, Bcl-(XL) and Bcl-2 suppression, and cytochrome c release, resulting in apoptosis [46-48]. MAB and antcin B (100 μM)-induced apoptosis is characterized by increased DNA fragmentation, cleavage of PARP, sub-G1 population appearance, and chromatin condensation [49]. Moreover, AC components induced NADPH oxidase-provoked oxidative stress [49,50]. Another purified AC component, antroquinonol, inhibited HepG2 (IC$_{50}$: 0.13 μM) and Hep3B (IC$_{50}$: 4.3 μM) cell growth [50,51]. Recently, Chiang et al. [52] demonstrated that antroquinonol induced HCC apoptosis through AMPK activation and mTOR translational pathway inhibition, and induced cell cycle arrest through downregulation and suppressed nuclear translocation of cyclin D1, cyclin E, Cdk4, and Cdk2. An AC ethyl acetate extract (IC$_{50}$: 42.5 μg/mL in HepG2 cells; 78.3 μg/mL in Hep3B cells), methanolic extract (IC$_{50}$: 49.5 μg/mL in HepG2 cells; 62.7 μg/mL in Hep3B cells), and ethanolic extract (IC$_{50}$: 54.2 μg/mL in HepG2 cells; 82.9 μg/mL in Hep3B cells) could also inhibit growth in a similar manner [4,46,48,53,54]. Apart from the direct cytotoxicity, cell cycle arrest can be used for controlling tumor growth. During the G1 phase of the cell cycle, the cells increase in size and synthesize essential proteins for the resting steps of the cell division process. It is important to control whether a cell goes into the S phase (DNA synthesis phase) or escapes from the cell cycle. An AC mycelium extract, 4-acetylantroquinonol B (EC$_{50}$: 0.1 μg/ mL), induced cell cycle arrest in HepG2 cells, indicating that cells were unable to divide. Western blotting analyses further showed that the protein expressions of different cell cycle-regulating proteins, CDK2 and CDK4, were decreased, while p21, p27, and p53 were increased in HepG2 cells after the AC treatment [55,56]. Similar to the case for the conventional anti-cancer drug cisplatin, tumor cell proliferation can be inhibited through induction of DNA damage, cell cycle arrest, and apoptosis. This suppresses the size of tumor tissues, and reduces the chance for subsequent metastasis [57,58].

Anti-angiogenic therapy has been explored as a therapeutic approach to HCC [59,60]. A series of angiogenic inhibitors, *e.g.*, bevacizumab (for blocking the angiogenic factor VEGF), endostatin, IFN-α, cyclooxygenase-2 (for inhibiting EC migration, tube formation, proliferation, and angiogenic factor production), interleukin-12 (for producing anti-angiogenic factors), and erlotinib and gefitinib (for inhibiting epidermal growth factor receptor tyrosine kinase activation) are currently in different phases of clinical trials [61].

An AC ethyl acetate extract and AC polysaccharides inhibited angiogenesis *in vitro* and *in vivo*. The ethyl acetate

extract was shown to inhibit cancer invasion of human liver cancer PLC/PRF/5 cells through suppression of angiogenic factor (e.g. VEGF) and extracellular digestive enzyme (e.g. MMP-2 and -9) production, and elevation of enzyme inhibitor (tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2) production in the concentration ranges of 10 to 40 μg/mL [47]. These results were confirmed in an animal model by Matrigel plug angiogenesis assays, in which the ethyl acetate extract (300 mg/kg) significantly suppressed tumor growth in nude mice [46]. The VGEF-induced tube formation and migration of ECs, and neovessel formation on chorioallantoic membranes were effectively inhibited by the AC polysaccharide extract [62,63]. Cheng et al. [64] demonstrated that the different fractionated AC polysaccharide extracts were able to inhibit VEGF protein expression, binding of VEGF to VEGFR-2, and VEGFR-2 phosphorylation, suggesting that inhibition of VEGF interactions with VEGF receptors is an anti-angiogenic mechanism of AC.

Anti-angiogenic therapy is particularly promising, because it is expected to minimize the devastating side effects, such as hair loss, bleeding, and immunosuppression, that are commonly associated with conventional chemotherapy. We previously found that many herbal medicines and natural compounds, such as ginsenosides, indirubin derivatives, and sinomenine, possess anti-angiogenic activities [65-70]. Therefore, AC may also contain anti-angiogenic components.

Effects on alcohol metabolism
Metabolism of alcohol and its related diseases
Chronic excessive consumption of alcohol often causes serious health problems, especially to the liver, such as cirrhosis and HCC [18,71]. According to the WHO, alcohol is the third largest risk factor for disease worldwide, and has been classified as a type I carcinogen by the IARC [72]. It has been estimated that alcohol leads to 2.5 million deaths each year, and that 320,000 of these deaths are in the age group of 15–29 years, representing 9% of all deaths in that age group [73]. Alcohol, a water-soluble small molecule, is easily absorbed into the bloodstream from the gastrointestinal tract. It cannot be stored, and is either metabolized or excreted [74,75]. More than 90% of absorbed alcohol is oxidized by hepatocytes, while the remainder is excreted through pulmonary respiration and urinary excretion [75,76]. There are three major pathways for metabolizing alcohol. The primary pathway of alcohol metabolism is the alcohol dehydrogenase (ADH)-mediated ethanol oxidation pathway [76,77]. ADH comprises a group of cytosolic enzymes that convert alcohol to acetaldehyde utilizing NAD^+ as a coenzyme, and the elements of this pathway are mainly located in the liver. At high alcohol concentrations (above 100 mg/dL) or during chronic alcohol consumption, the ADH pathway may become saturated through NAD^+ depletion. When this happens, the secondary pathway, the microsomal ethanol oxidizing system, is used to break down alcohol [76,77]. In this system, cytochrome P450 enzymes such as CYP2E1, CYP1A2, and CYP2A4 metabolize alcohol to acetaldehyde via NADPH as a coenzyme instead of NAD^+ [76,78]. The third pathway is the hydrogen peroxide (H_2O_2)-generating system in peroxisomes [76], which oxidizes alcohol to acetaldehyde and water molecules [75,77]. The acetaldehyde product is further oxidized by aldehyde dehydrogenase (ALDH) to acetate, and subsequently processed in the citric acid cycle. The final products are carbon dioxide, water, and ATP. Through the ADH and ALDH-mediated reactions, NAD^+ is used up in the form of a coenzyme, and NADH is generated. This increased ratio of NADH:NAD^+ redox potential in the liver inhibits mitochondrial β-oxidation of fatty acids and reduces lipid expenditure, resulting in lipogenesis and lipid accumulation in hepatocytes [79,80]. The severity of this alcoholic liver disease (ALD) depends on the amount, frequency, and duration of alcohol consumption, and can further develop into more complicated liver damage states, including fibrosis, steatohepatitis, cirrhosis, and cancer [81-83].

Anti-alcoholic activity
Different types of AC preparations, including ethanolic extracts, aqueous extracts, fermented filtrates, and crude powder, can protect the liver against alcohol damage. Ethanol-induced ALD rats were orally treated with AC ethanolic extracts at a range of 0.25–1.25 g/kg. Hepatic damage was assessed using serum markers of liver damage, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) [84]. When liver cells are damaged, these enzymes are released into the bloodstream where they can be detected with biochemical or immunological assay kits. The AC extracts could prevent ethanol- and carbon tetrachloride (CCl_4)-induced elevations of the AST, ALT, and ALP levels in the rat blood serum [85,86], indicating that AC conferred protection against chemical-induced liver damage. Moreover, the AC ethanolic extract could normalize the alcohol-induced increase in malondialdehyde, and decreases in glutathione peroxidase, reductase, and glutathione levels in the liver [86,87]. In the CCl_4-induced ALD model, AC extracts could restore the reduction in the hepatic glutathione content and catalase (CAT) activity, and prevent free radical formation and lipid peroxidation [88]. These *in vivo* data were validated in a HepG2 cellular model by Kumar et al. [89], who showed that the antroquinonol from the AC ethanolic extract could protect ethanol-induced HepG2 cells against heme oxygenase 1, NF-E2 released factor (Nrf-2), and mitogen-activated protein kinase activation (MAPK).

An AC fermented filtrate protected HepG2 cells against damage caused by CCl_4 [90] and H_2O_2 [91]. Song et al. [91] demonstrated that the AC fermented filtrate (0.05–0.5 mg/mL) suppressed lipid peroxidation in H_2O_2-induced HepG2 cells. The AC fermented filtrate restored the CCl_4-induced increases in ALT, AST, lipid peroxidation, liver lesions, neutrophil infiltration, hydropic swelling, and necrosis, and the reductions in glutathione peroxidase, reductase, and transferase in animal tests [91]. The liver damage caused by chronic alcohol consumption was found to be reduced by an AC crude powder. Chen et al. [85] demonstrated that AC crude powder-treated rats had relatively smaller livers, less lipid accumulation, and lower AST, ALP, and serum alcohol levels. In addition, their serum and hepatic MMP-9 activities, and TNF-α, krueppel-like factor-6 (KLF-6), and transformation growth factor-β (TGF-β) gene expressions were downregulated. Moreover, the AC powder could enhance the metabolism of alcohol by increasing the CAT and ALDH activities [85]. Chen et al. [92] further demonstrated that the AC crude powder induced downregulation of 3-hydroxy-3-methylglutaryl-CoA reductase, sterol regulatory element-binding protein-1c, acetyl-CoA carboxylase, fatty acid synthase, and malic gene expression, and upregulation of low-density lipoprotein receptor and peroxisome proliferator-activated alpha gene expression [92]. A dried powder from fermented AC mycelium (0.34–0.57 g/kg) could protect the rat liver against damage from alcohol, and significantly suppress the alcohol-induced increases in glutamate-pyruvate aminotransferase, glutamate-oxaloacetate aminotransferase, superoxide dismutase, and CAT activities. Histological data validated the AC protective effects by showing a reduction in alcohol-induced lipid vacuole accumulation, and hydropic degradation of hepatocytes [93].

Conclusions
Hepatoprotective activities, including anti-hepatitis, anti-hepatocarcinoma, and anti-alcoholism, of AC have been demonstrated *in vitro* and *in vivo*. However, many of the underlying mechanistic actions, such as the pharmacokinetics and pharmacodynamics, remain unclear. Furthermore, human and clinical trials are required for a full evaluation of this valuable medicinal mushroom for potential clinical applications.

Abbreviations
AC: Antrodia cinnamomea; FDA: Food and Drug Administration; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HCC: Hepatocellular carcinoma; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; IFN-α: Interferon-α; WHO: World Health Organization; IARC: International Agency for Research on Cancer; TNF-α: Tumor necrosis factor; IL-1β: Interleukin-1β; IL-6: Interleukin-6; VEGF: Vascular endothelial cell growth factor; FGF-2: Fibroblast growth factor-2; EC: Endothelial cell; VEGFR-1: VEGF receptor 1; VEGFR-2: VEGF receptor 2; MMP: Matrix metalloproteinase; MAA: Methyl antcinate A; MAB: Methyl antcinate B; TIMP-1: Tissue inhibitor of metalloproteinases-1; TIMP-2: Tissue inhibitor of metalloproteinases-2; ADH: Alcohol dehydrogenase; H_2O_2: Hydrogen peroxide; ALDH: Aldehyde dehydrogenase; ALD: Alcoholic liver disease; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; CCl_4: Carbon tetrachloride; CAT: Catalase; Nrf-2: NF-E2 released factor; MAPK: Mitogen-activated protein kinase activation; ALDH: Aldehyde dehydrogenase.

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