Use of *Ganoderma lucidum* fermented product for improving alcoholic liver disease, alcoholic fatty liver and diseases associated with inflammation.

BACKGROUND OF THE INVENTION

1. Field of the invention

The present invention relates to a use of *Ganoderma lucidum* fermented product for improving alcoholic fatty liver and alcoholic liver disease and inhibiting inflammatory responses.

2. Description of the prior art

Liver disease is resulted when the liver is injured and unable to perform its normal functions. Common causes of liver disease include virus, drug and alcohol, and alcohol consumption has always been the typical cause of alcoholic liver disease (ALD).

ALD is usually divided into several stages based on progression and the degree of severity of the disease: the first stage is accumulation of excess fat in the liver which is called fatty liver, followed by injury and inflammation of the liver which is called hepatitis and fibrosis of the liver which is called liver cirrhosis, and finally the disease may become life-threatening or proceed to liver cancer. Therefore, further progression of liver disease can be prevented by effective control of fatty liver and hepatitis.

The mechanism of alcohol-induced liver disease: long-term

consumption of alcohol leads to alcoholic liver disease and the first stage of ALD is fatty liver. The characteristic of fatty liver is accumulation of excess fat in the liver followed by the symptoms of hepatitis and abnormal liver function tests, for example, elevated aspartate aminotransferase (AST) and elevated alanine aminotransferase (ALT) levels accompanied by abnormal liver functions, such as hyperlipidemia and poor blood glucose control. Although the pathological mechanism of ALD caused by alcohol consumption has not yet been clarified, alcohol has been proven to induce fatty liver and hepatitis through the following pathways: (1) during the metabolic process of conversion of alcohol into acetaldehyde and acetic acid, alcohol inhibits mitochondrial function and consequently leads to abnormal lipid metabolism and delayed output of triglyceride and cholesterol in liver cells, resulting in accumulation of excess fat in the liver. Such fatty lesion may further induce inflammatory responses and hepatic necrosis. (2) Peroxides will be generated during the process of conversion of excess alcohol into acetaldehyde in the liver, which will trigger the chain reactions of free radicals and produce excess peroxides as well as deplete hepatic antioxidants and hepatic antioxidant enzymes. In addition, acetaldehyde can promote oxidation which will cause peroxidation of lipids and lead to liver cell injury. (3) Alcohol will destroy intestinal mucosal barrier and allow intestinal microorganisms to pass the intestinal mucosa and enter the liver via hepatic portal vein, which will subsequently stimulate Kupffer cells

(the specialized macrophages located in the liver) to release excess inflammatory cytokines, e.g. TNF-α (tumor necrosis factor-α) and interleukin-6 (IL-6), resulting in liver injury and inflammation. In addition to the three major mechanisms mentioned above, alcohol metabolism will also induce hypoxia in the liver as well as hepatic microcirculation disorders, which will help the development of alcoholic liver disease to a certain extent.

The fruiting body of *Ganoderma lucidum* is a common fungal medicinal material in East Asia and has the functions of nourishing and strengthening, prolonging life as well as enhancing immunity and is widely used in traditional Chinese medicine. In recent years, the technology of deep liquid fermentation has been widely used for mass production of the mycelium and mycelium ferment filtrate of *Ganoderma lucidum* to meet the market demand for *Ganoderma lucidum* products. When compared with the traditional culture methods, this production method dramatically shortens the time required for production of *Ganoderma lucidum*. The mycelium and ferment filtrate of *Ganoderma lucidum* obtained by liquid fermentation are different from the traditionally edible fruiting bodies of *Ganoderma lucidum*, and the parts, appearance, chemical activity (see references 1-2) as well as the pharmacological activity are all different (see reference 3-4). Moreover, the overall physiological functions and activities also vary significantly from the traditional fruiting bodies of *Ganoderma lucidum*. Therefore, the function of the *Ganoderma lucidum* fermented product is not

the same as the fruiting body of *Ganoderma lucidum*.

From previous studies of *Ganoderma lucidum*, the extract or triterpenoids of *Ganoderma lucidum* can protect liver cells *in vitro* (see references 5-9). In addition, past literatures have also reported that the fruiting body, mycelium, fermented liquid or extract of *Ganoderma lucidum* can protect liver cells in the model of nonalcoholic fatty liver disease, e.g. chemical liver injury model (induced by carbon tetrachloride or tert-butyl hydroperoxide, see references 10-14), hepatitis B model (see reference 15), immunity model (induced by BCG or combination of BCG and lipopolysaccharide), catabolic pathway interference model (induced by galactosamine, alpha-amanitin, see references 20-21), and high-fat diet model (see references 22-26). Moreover, other studies also have shown that the polysaccharides and spore powder of *Ganoderma lucidum* fruiting bodies have hepatoprotective effect in acute alcoholic liver injury (see references 27-28). Though *Ganoderma lucidum* produced by different methods has been demonstrated to have hepatoprotective effect in several models, *Ganoderma lucidum* fermented products have not yet been proved to have the functions of improving liver injury or fatty liver induced by alcohol.

The Republic of China Patent (Publication No. 201221134)

discloses a composition for improving liver function and reducing liver injury and *Ganoderma lucidum* is simply one of the ingredients of this composition. In addition, the *Ganoderma lucidum* described in the invention refers to the fruiting bodies of *Ganoderma lucidum* and the experimental model utilized toxins for induction of the HepG2 cell line, such as carbon tetrachloride and chloroform, followed by comparison of cell survival rates.

The China patent (Publication No. CN 103272146 B) reveals a pharmaceutical composition for prevention of alcoholic fatty liver and *Ganoderma lucidum* is simply one of the ingredients of this composition, and the *Ganoderma lucidum* specified in the invention is the extract of its fruiting bodies.

The Republic of China Patent (Patent No. I397415) discloses an anti-inflammatory and hepatoprotective formulation, wherein the *Ganoderma lucidum* immunoglobulin was generated by gene recombination and a nucleotide fragment of *Ganoderma lucidum* was cloned into yeast to recombine the genes for mass production of the *Ganoderma lucidum* immunoglobulin by fermentation.

Some studies have shown that *Ganoderma lucidum* has antioxidative effect in chemical, cellular anabolic pathway interference, radiation and edible peroxide oil models (see references 29-32). However, the liquid fermented products of

Ganoderma lucidum have not yet been proved to have the capacity to improve reduction of antioxidative activity caused by alcohol.

The Japanese Patent (Publication No. 2004-131482) discloses that the spores of *Ganoderma lucidum* have antioxidative effect and can delay the aging process as well as ameliorate the menopausal symptoms in men.

Due to the complexity of the pathological mechanism of alcoholic liver disease, it is difficult to replace such mechanism with relatively simple models such as chemical models and the ALD model must be used to evaluate the effect of a substance on improving liver injury, fatty liver and reduced antioxidative effect caused by alcohol. In addition, though the acute ALD model uses alcohol to induce liver injury and fatty liver, excess alcohol is given in a single dose via gavage, which is different from normal drinking behaviors of people and thus is not suitable for evaluation of the effect on improving ALD.

In normal situations, the body will secrete adequate amount of pro-inflammatory cytokines to regulate health, but in case of infection, pathogenic bacteria will produce excess endotoxins which can activate immunity after being decomposed, e.g. lipopolysaccharide (LPS) and peptidoglycan, such activation will subsequently activate macrophages and monocytes. On the other

hand, when the immune system is activated abnormally, excess pro-inflammatory cytokines will be made and such excess pro-inflammatory cytokines, e.g. TNF-α, interleukin-6 (IL-6), and interleukin-8 (IL-8), will cause damage to the body. TNF-α and IL-6 will initiate and escalate inflammatory responses and IL-8 will induce neutrophils, macrophages, basophils, and T cells to move toward the sites of inflammation and further escalate the inflammatory responses in tissues and exacerbate injury. TNF-α, IL-6 and IL-8 are the major regulators in inflammatory responses and thus can be used as the indicators for inflammation.

Abnormal secretion of TNF-α and IL-6 by macrophages will lead to many inflammatory disorders such as hepatitis, nephritis, arthritis and sepsis. For example, the concentrations of TNF-α and IL-6 in the patients with viral hepatitis or chronic hepatitis are significantly higher than healthy people (see references 33-34), whereas neutralization or reduction of TNF-α and IL-6 secretion will improve the degree of severity of inflammatory diseases. For example, infliximab, a drug for treating hepatitis, is an anti-TNF-α drug (see reference 35). Hence, inhibition of TNF-α and IL-6 secreted by macrophages will improve inflammatory diseases such as hepatitis, nephritis, arthritis and sepsis.

Abnormal secretion of excess IL-8 by the intestinal tract will

also cause intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis. The intestinal IL-8 produced by the patients with colitis is significantly higher and the amount of secreted IL-8 as well as the degree of severity of colitis of these patients are positively correlated with the number of entered inflammatory cells (see references 36-37), indicating IL-8 will promote intestinal inflammation while the antibodies capable of neutralizing IL-8 can effectively inhibit invasion of the inflammatory cells into the intestinal tract (see reference 38). Therefore, inhibition of intestinal IL-8 expression will improve colitis.

Previous report has indicated that the fruiting bodies and solid fermented products of *Ganoderma lucidum* can inhibit production of the inflammatory mediator, TNF-α (see references 39-42), but some other studies have reported the fruiting bodies and solid fermented products of *Ganoderma lucidum* have a completely opposite effect and will promote secretion of the inflammatory mediators such as TNF-α (see references 4, 43-44), suggesting different species or different parts of *Ganoderma lucidum* or different production methods will produce different metabolites and thus their effects on inflammatory responses will be different. Nonetheless, no study has reported that the liquid

fermented product of *Ganoderma lucidum* has anti-inflammatory effect.

In summary, though *Ganoderma lucidum* or its liquid fermented product has been utilized to improve fatty liver and liver injury, the liquid fermented product of *Ganoderma lucidum* has not been used to improve fatty liver, liver disease, reduced hepatic antioxidants and reduced activities of hepatic antioxidant enzymes caused by alcohol and to inhibit inflammatory responses. Therefore, deep liquid fermentation of *Ganoderma lucidum*, including its mycelium and fermented liquid, and improving fatty liver, deterioration of liver functions and reduced antioxidative effect caused by alcohol as well as inhibition of inflammatory responses have become the important tasks of this invention.

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SUMMARY OF THE INVENTION

Though previous studies have reported using the fruiting bodies or liquid fermented products of *Ganoderma lucidum* to improve nonalcoholic fatty liver and liver damage, the liquid fermented products of *Ganoderma lucidum* have not yet been utilized to improve fatty liver, liver disease and reduced hepatic antioxidant effect caused by alcohol and to inhibit systematic and intestinal inflammation. Hence, deep liquid fermentation of *Ganoderma lucidum*, including its mycelium and fermented liquid, and improvement of fatty liver, deterioration of liver function and reduced antioxidative effect caused by alcohol as well as inhibition of inflammatory responses have become the important tasks of this invention.

In one aspect, present invention provides a use of a composition for manufacture of a health food or medicinal product for treating liver diseases caused by alcohol, said composition is comprising of the liquid fermented products of *Ganoderma lucidum* produced by liquid culture.

According to the invention, the liver diseases refer to alcoholic liver disease and alcoholic fatty liver caused by alcohol,

reduced concentration of hepatic antioxidant caused by alcohol, or reduced activity of hepatic antioxidant enzymes caused by alcohol.

According to the invention, alcoholic liver disease refers to abnormally elevated liver indicators or abnormal liver function.

According to the invention, abnormal liver indicators refer to elevated serum AST level or elevated serum ALT level.

According to the invention, abnormal liver functions comprise elevated triglycerides, elevated cholesterol or elevated blood glucose level.

According to the invention, alcoholic fatty liver includes increase of triglycerides, cholesterol or total fat in the liver.

According to the invention, the antioxidant refers to glutathione.

According to the invention the antioxidant enzymes include SOD (Superoxide dismutase), Catalase, GSH-Rd (GSH Reductase), GSH-Px (GSH Peroxidase) or GST (Glutathione S-transferase).

In another aspect, present invention provides a use of a composition for manufacture of a health food or medicinal product for improving macrophage-related inflammatory diseases and said composition is comprising of liquid fermented products of *Ganoderma lucidum* obtained from liquid culture.

According to the invention, macrophage-related inflammatory diseases include diseases related to increased secretion of TNF-α and IL-6, including hepatitis, nephritis, sepsis, arthritis or periodontitis.

In the other aspect, present invention provides a use of a composition for manufacture of a health food or medicinal product for improving intestinal cell-related inflammatory diseases and said composition is comprising of liquid fermented products of *Ganoderma lucidum* obtained from liquid culture.

According to the invention, the intestinal cell-related inflammatory diseases refer to the diseases related to increased secretion of IL-8, including Crohn's disease, ulcerative colitis or Rotavirus colitis.

According to the invention, the features of the liquid fermented product of *Ganoderma lucidum* include polysaccharides with a molecular weight of 23~826 kDa and polysaccharides with a molecular weight of 827~over 1,600 kDa.

According to the invention, said composition can further contain a flavoring substance or adjuvant and said flavoring substance is comprising of sugars, acids, fruit juices, condiments or water.

According to the invention, the sugars refer to substances that

can provide sweetness, including but not limited to, sucrose, dextrose, fructose or sucrose; wherein the acids refer to substances that can provide sourness, including but not limited to, citric acid, malic acid, tartaric acid or phosphoric acid; wherein the fruit juices refer to substances commonly used in modification of beverage flavors, including but not limited to, apple juice, orange juice or grape juice; wherein the condiments refer to any substance that can change the odor, including but not limited to, synthetic flavors, vanilla seeds or mint extracts.

According to the invention, said composition can be prepared in various dosage forms, including liquid nutrient supplements, liquors, beverages, powders, capsules, tablet, pills, granules, jellies, chewing gums, or other dosage forms that may be taken orally.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is the schematic diagram of the process of liquid fermentation and preparation of the test samples for *Ganoderma lucidum*

Fig. 2 is the H&E stain results of the liver cells of mice. The effect of the fermented product of *Ganoderma lucidum* on fat accumulation in the liver of mice caused by alcohol.

Fig. 3 shows the peak retention time of the calibration curve of the standard and the fermented liquid of *Ganoderma lucidum* analyzed by using the RI (Refractive index) detector of HPLC and Gel permeation chromatography.

Fig. 4 shows the chromatograms of the fermented liquid of *Ganoderma lucidum* analyzed by using the RI (Refractive index) detector of HPLC with Gel permeation chromatography.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention will be better elucidated when read in conjunction with the following examples; however, it should be understood that the invention is not limited to the preferred embodiments shown.

Example 1 Animal studies for examination of effective improvement of alcoholic liver disease and alcoholic fatty liver caused by alcohol, reduced hepatic antioxidant concentrations and reduced activities of the hepatic antioxidant enzymes.

The experimental method is using the Lieber-DeCarlialcohol liquid feed which replaces carbohydrates by alcohol which contains the same amount of calories as the basic diet and the alcohol accounts for 36% of the total calories of the feed to mimic the

alcohol consumption habit of humans and induce alcoholic fatty liver and alcoholic liver disease. Said model is a major animal model used currently for induction of alcoholic fatty liver and ALD. To simulate the long-term alcohol consumption condition in humans, the animal study lasted 8 weeks and the study animals were male C57BL/6J mice aged 8-10 weeks with a weight around 20 g. The mice were divided into 4 groups, 12 mice per group. Group 1: the control group is given pure water through gavage in addition to normal liquid feed; Group 2: the alcohol group is given pure water through gavage in addition to alcoholic liquid feed; Group 3: the *Ganoderma lucidum* low-dose group is given 0.513 mg/g BW of freeze-dried powder of *Ganoderma lucidum* fermented liquid in addition to alcoholic liquid feed; Group 4: the *Ganoderma lucidum* high-dose group is given 2.05 mg/g BW of freeze-dried powder of *Ganoderma lucidum* fermented liquid in addition to alcoholic liquid feed. The feeding condition was monitored throughout the entire study period and the fasting biochemical values, hepatic fat accumulation, hepatic antioxidant level and activities of the hepatic antioxidant enzymes were analyzed before and after the experimental feeding.

The freeze-dried powder of *Ganoderma lucidum* was produced by addition of sugars, acids, fruit juices, condiments and water to the fermented liquid of *Ganoderma lucidum* obtained from

liquid culture (as shown in Fig. 1) and freeze drying of the composition will give rise to the test substance, the freeze-dried powder of *Ganoderma lucidum* fermented liquid.

The sugar refers to substances that can provide sweetness, including but not limited to, sucrose, glucose, fructose, lactose, inulin, sorbitol, xylitol, fructo-oligosaccharides, aspartame, sucralose and the like. The acid refers to substances that can provide sourness, including but not limited to, citric acid, malic acid, phosphoric acid, tartaric acid and the like. The juice refers to substances commonly used in modification of beverage flavors, including but not limited to, apple juice, orange juice, peach juice, grape juice, mango juice, pineapple juice, grapefruit juice, lemon juice, kiwi juice, prune juice, and strawberry juice.

The intake amount of each group of mice during the study period

From Table 1, the intake amount for each group did not change during the study period, indicating the *Ganoderma lucidum* composition and alcohol did not affect the intake amount and total caloric intake of the mice.

Table 1 The intake amount of animal feed for each group during the

study period

Group	Week 1	2	3	4
Control group (g)	20.8 ± 1.0[a]	20.7 ± 1.2[a]	22.5 ± 1.8[a]	22.6 ± 1.6[a]
Alcohol group (g)	20.8 ± 1.3[a]	20.7 ± 1.3[a]	22.9 ± 1.2[a]	22.8 ± 1.1[a]
Ganoderma lucidum low-dose group (g)	20.7 ± 1.4[a]	20.6 ± 1.4[a]	22.6 ± 1.4[a]	22.4 ± 1.3[a]
Ganoderma lucidum high-dose group (g)	20.7 ± 1.3[a]	20.5 ± 1.2[a]	22.7 ± 1.4[a]	22.6 ± 1.4[a]

Group	Week 5	6	7	8
Control group (g)	24.7 ± 1.6[a]	24.8 ± 1.4[a]	26.5 ± 1.2[a]	26.7 ± 1.2[a]
Alcohol group (g)	24.8 ± 1.5[a]	24.8 ± 1.4[a]	26.2 ± 1.0[a]	26.4 ± 1.1[a]
Ganoderma lucidum low-dose group (g)	24.4 ± 1.9[a]	24.8 ± 1.3[a]	26.3 ± 1.1[a]	26.5 ± 1.2[a]
Ganoderma lucidum high-dose group (g)	24.5 ± 1.6[a]	24.4 ± 1.5[a]	26.3 ± 1.1[a]	26.4 ± 1.2[a]

The effect of *Ganoderma lucidum* fermented product on biochemical markers of liver function

The biochemical markers of each group at the end of 8-week feeding are shown in Table 2. The AST and ALT markers of liver damage, total cholesterol as well as the total triglyceride were all significantly higher in the alcohol group. In addition, the blood glucose level was also significantly higher, indicating long-term consumption of alcohol will lead to liver injury and influence the liver to maintain normal blood cholesterol and blood glucose levels. The

ALT markers of the low-dose and high-dose group fed with freeze-dried *Ganoderma lucidum* fermented liquid showed no significant difference when compared with the control group (p>0.05), suggesting intake of the *Ganoderma lucidum* sample can restore the ALT levels of mice to normal range. The serum AST, total cholesterol, total triglyceride and blood glucose levels were also reduced significantly with increasing dose of *Ganoderma lucidum*.

Table 2 Serum biochemical markers of each group after the end of 8-week feeding

	Control	Alcohol	*Ganoderma lucidum* low-dose	*Ganoderma lucidum* high-dose
AST (U/L)	59.1 ± 12.6^{b}	216.6 ± 65.1^{a}	109.3 ± 15.0^{c}	96.4 ± 13.9^{c}
ALT (U/L)	31.3 ± 9.0^{b}	50.8 ± 12.4^{a}	34.8 ± 8.0^{b}	31.3 ± 7.3^{b}
Total cholesterol (mg/dL)	82.3 ± 8.4^{b}	113.8 ± 11.5^{a}	100.5 ± 10.8^{c}	95.8 ± 7.6^{c}
Total triglyceride (mg/dL)	72.3 ± 9.3^{b}	105.0 ± 12.6^{a}	87.5 ± 14.2^{c}	75.4 ± 10.6^{bc}
Glucose (mg/dL)	117.8 ± 23.0^{b}	245.0 ± 32.3^{a}	205.6± 37.2^{c}	179.5 ± 42.6^{c}

Long-term consumption of alcohol will increase hepatic accumulation of cholesterol and liver triglyceride. As shown in Table 3, the *Ganoderma lucidum* high-dose and low-dose groups can effectively reduce hepatic fat accumulation and improve the symptoms of fatty liver and fat accumulation reduced with increasing dose of *Ganoderma lucidum*. It is apparent from Fig. 2 that the

histopathological sections of the mice fed with *Ganoderma lucidum* show decreased fat accumulation (oil droplets) and fat accumulation decreases with increasing dose of *Ganoderma lucidum*. The results shown above indicate the high-dose group has a better effect on reducing fat accumulation, suggesting the *Ganoderma lucidum* liquid fermented composition has the function of improving alcoholic fatty liver.

Table 3 The effects of *Ganoderma lucidum* on change of biochemical markers induced by alcohol.

	Control group	Alcohol group	*Ganoderma lucidum* low-dose group	*Ganoderma lucidum* high-dose group
Liver weight (mg/g mice)	38.7 ± 2.3^{a}	40.0 ± 1.7^{a}	40.6 ± 1.8^{a}	40.1 ± 2.6^{a}
Liver cholesterol (mg/g liver weight)	99.9 ± 18.4^{b}	228.2 ± 18.4^{a}	196.0 ± 32.8^{c}	179.4 ± 37.4^{c}
Liver triglyceride (mg/g liver weight)	83.0 ± 16.05^{b}	233.6 ± 40.3^{a}	151.5 ± 28.1^{c}	141.5 ± 16.2^{c}
Fatty liver score[#]	0 ± 0^{b}	2.17 ± 0.58^{a}	1.00 ± 0.74^{c}	$0.67 \quad 0.65^{c}$

Determination of the concentration of the antioxidant and the activities of the antioxidant enzymes in frozen mouse liver after thawing. As shown in Table 4, in addition to reducing the concentration of hepatic antioxidant GSH (Glutathione), long-term consumption of alcohol also decreases the activities of antioxidant enzymes, including GSH-Rd (GSH Reductase), GSH-Px (GSH Peroxidase), GST (Glutathione S-transferase), SOD (Superoxide

dismutase) and Catalase, and reduces hepatic antioxidation and detoxification. The activities of the antioxidants such as GSH and SOD in the livers of the mice fed with high- and low-dose *Ganoderma lucidum* showed an increase and among which the high-dose group has a better effect and the levels of GSH and GSH-RD and the activity of GST of the high-dose group have recovered to the levels of the control group, suggesting the liquid fermented composition of *Ganoderma lucidum* disclosed in the invention has the function to promote the antioxidative system after alcohol consumption and to protect the liver from oxidative stress.

Table 4 Changes of the activities of hepatic antioxidant enzymes and the levels of antioxidants

	Control	Alcohol	*Ganoderma lucidum* low dose	*Ganoderma lucidum* high dose
GSH (μg/mL)	279± 24^{a}	117 ± 28^{c}	217 ± 58^{b}	275 ± 27^{a}
GSH-Rd (nmol NADPH/min/mg protein)	0.054±0.009^{a}	0.036±0.003^{b}	0.039±0.004^{b}	0.049±0.007^{a}
GSH Px (nmol NADPH/min/mg protein)	0.055± 0.011^{a}	0.031±0.007^{b}	0.035±0.008^{b}	0.046± 0.005^{c}
GST (nmol/min/mg protein)	1.79± 0.15^{a}	1.43± 0.14^{b}	1.46± 0.12^{b}	1.76± 0.12^{a}
SOD (U/mg protein)	288± 16^{a}	190± 53^{b}	217± 42^{bc}	245± 36^{c}
Catalase (U/mg protein)	386± 45^{a}	195± 56^{b}	256± 32^{c}	285± 38^{c}

Example 2 Studies of the effect of *Ganoderma lucidum* on inhibition

of the pro-inflammatory cytokines secreted by Raw264.7 macrophages and HT-29 human colon carcinoma cells.

In this example, Raw264.7 macrophages and HT-29 human colon carcinoma cells were co-cultured with the fermented liquid culture of *Ganoderma lucidum*, and the supernatant was collected for analysis of the concentrations of cytokines by using the OptEIATM Human ELISA Set (BD Biosciences, CA., USA) with an ELISA reader (Model 680; BIO-RAD) to evaluate the effect of *Ganoderma lucidum* on inflammatory responses.

Adjust and seed Raw264.7 macrophages at a density of 2×10^5 cells/ml and culture the cells at 37℃, 5%CO_2 for 48 hours, followed by addition of the alcoholic extract of *Ganoderma lucidum* (as shown in the preparation procedures of Fig.1) and incubation for additional 24 hours. The concentration of the cytokine contained in the supernatant is shown in Table 5. From the results, the alcoholic extract of *Ganoderma lucidum* significantly inhibits TNF-α secreted by macrophages and the inhibition effect increases with increasing concentration of the alcoholic extract of *Ganoderma lucidum*. The preferred conditions for preparation of the alcoholic extract of *Ganoderma lucidum* is to add 95% ethanol to the mycelium powder of *Ganoderma lucidum* at a weight ratio of 20:1 (w:w), mix well and stir

the mixture continuously at room temperature for 48 hours, followed by extraction for 1 hour at 65°C, collection of the supernatant after cooling and centrifugation to give test sample 1; add 95% ethanol to the centrifuged precipitate at a weight ratio of 20:1 (w:w) and mix thoroughly followed by extraction for 1 hour at 65°C, collection of the supernatant after cooling and centrifugation to give test sample 2; mix test sample 1 and test sample 2 and use an evaporator to remove alcohol and water and store the alcoholic extract of *Ganoderma lucidum* mycelium at 4°C for future use.

Table 5 The effect of co-culture of the *Ganoderma lucidum* alcoholic extract and RAW246.7 cells on TNF-α secretion after 24-hr incubation.

Group	Mean± SD (pg/ml)
Control group	6659 ±1006
Ganoderma lucidum alcoholic extract 10 mcg/mL	673 ±164
Ganoderma lucidum alcoholic extract 100 mcg/mL	253 ± 42
Ganoderma lucidum alcoholic extract 1mg/mL	202 ± 4
Ganoderma lucidum alcoholic extract 10mg/mL	33.3 ±7.0

Seed macrophages onto a 24-well plate at a density of $2x10^5$ cells and culture at 37℃, 5%CO_2 for 48 hours, followed by addition of the *Ganoderma lucidum* alcoholic extract or fermented liquid of *Ganoderma lucidum* (as shown in the preparation procedures of Fig.1, hereinafter *Ganoderma lucidum* water extract), LPS and Raw264.7 cells and incubate the cells for additional 24 hours. The IL-

6 levels in the supernatants are shown in Table 6 and Table 7. The preferred conditions for preparation of *Ganoderma lucidum* is as described above. From the results, both the *Ganoderma lucidum* alcoholic extract and water extract can significantly inhibit secretion of IL-6 by macrophages and the inhibition effect increases with increasing concentration of the test sample.

Table 6 The effect of co-culture of the *Ganoderma lucidum* alcoholic extract and RAW246.7 cells on IL-6 secretion induced by LPS after 24-hr incubation.

Group	Mean ± SD (pg/ml)
Negative control group	6.64 ± 0.04
LPS control group	7662 ± 237
LPS + *Ganoderma lucidum* alcoholic extract 10 mcg/mL	328 ± 48
LPS + *Ganoderma lucidum* alcoholic extract 100 mcg/mL	267 ± 2
LPS +*Ganoderma lucidum* alcoholic extract 1mg/mL	3.55 ± 0.81
LPS + *Ganoderma lucidum* alcoholic extract 10mg/mL	1.39 ± 0.05

Table 7 The effect of co-culture of the *Ganoderma lucidum* water extract and RAW246.7 cells on IL-6 secretion induced by LPS after 24-hr incubation.

Group	Mean ± SD (pg/ml)
Negative control group	6.18 ± 1.12
LPS control group	7468 ± 403
LPS + *Ganoderma lucidum* water extract 40 mcg/mL	753 ± 0
LPS + *Ganoderma lucidum* water extract 400 mcg/mL	599 ± 132
LPS + *Ganoderma lucidum* water extract 40 mg/mL	402±16

When macrophages are activated abnormally and release

excess pro-inflammatory cytokines such as TNF-α and IL-6 in experimental simulations of inflammatory diseases such as hepatitis, nephritis, arthritis or periodontitis by using Raw264.7 cells, both the *Ganoderma lucidum* alcoholic and water extracts can efficiently inhibit TNF-α and IL-6 secretion, indicating *Ganoderma lucidum* liquid fermented products have the function of improving the inflammatory diseases associated with macrophages.

Detach HT-29 cells from culture dishes by using Trypsin EDTA and separate the cells into single-cell suspension and seed the cells onto a 24-well plate at a density of $3x10^5$ cells per well and incubate the plate at 37℃, 5%CO_2 for 48 hours to allow attached growth, followed by addition of the *Ganoderma lucidum* water extract before cocultivation for additional 24 hours. Examination of IL-8 secreted by HT-29 cells and the results are shown in Table 8. The *Ganoderma lucidum* alcoholic and water extracts can significantly inhibit secretion of the inflammatory factor IL-8 by the intestinal cells and the inhibition effect increases with increasing concentration of the *Ganoderma lucidum* water extract.

Table 8 The effect of co-culture of the *Ganoderma lucidum* water extract and HT-29 cells on IL-8 secretion after 24-hr incubation.

Group	Mean ± SD (pg/ml)
Control group	122 ± 11
Ganoderma lucidum water extract 40	8.88±10.49

mcg/mL	
Ganoderma lucidum water extract 400 mcg/mL	2.96 ± 2.39
Ganoderma lucidum water extract 40 mg/mL	2.39 ±0.40
Ganoderma lucidum water extract 400 mg/mL	0.99 ±0.57

When HT-29 colon cancer cells are activated abnormally and release excess pro-inflammatory cytokines such as IL-8 in experimental simulations of intestinal inflammatory diseases such as Crohn's disease, ulcerative colitis or Rotavirus colitis by using HT-29 cells, the *Ganoderma lucidum* water extract can efficiently inhibit IL-8 secretion, indicating *Ganoderma lucidum* liquid fermented product has the function of improving diseases associated with intestinal inflammation.

Example 3 Analysis of the molecular weights and ratios of the polysaccharides in *Ganoderma lucidum* liquid fermented product

Previous studies have reported the effective ingredients of *Ganoderma lucidum*, for example, *Ganoderma lucidum* acid, polysaccharides and small peptides, but the *Ganoderma lucidum* fermented liquid barely contains *Ganoderma lucidum* acid. Past literatures also have suggested the *Ganoderma lucidum* fermented liquid contains functional small peptides. The polysaccharides of *Ganoderma lucidum* have been proven to have positive effects in a

non-alcoholic liver protection model and therefore the ingredients that have the alcoholic protective effect in the fermented liquid may be polysaccharides. The structure of natural polysaccharides is usually complex but the molecular weight of a polysaccharide is associated with the strength of different functions and thus the molecular weight of a polysaccharide is an important feature of polysaccharides. Hence, HPLC and GPC (gel permeation chromatography) were used to analyze the distribution of the molecular weight of the *Ganoderma lucidum* fermented liquid via a RI (Refractive index) detector.

Experimental conditions:

1. Sample pre-treatment: *Ganoderma lucidum* fermented liquid (prepared by following the procedures specified in Fig.1) is diluted 20-fold in de-ionized water before being placed in a dialysis bag (MWCO=6-8 kD, Spectra/Por 1 Standard RC Dry Dialysis Tubing, Spectrum) for continuous dialysis in flowing water for 24 hours. Filter the dialysate with a 0.45μM filter and analyze the sample.

2. Equipment:
HPLC system: HEWLETT PACKARD series 1100, Agilent
Detector: RI detector, Waters 2414, Waters
Standards: Pullulan with a molecular weight of 20, 50, 100, 200, 400, 800 and 1,600 kDa.
GPC column: OHpak SB-805 HQ ($8mm^{ID}$*$300mm^{L}$, 13um)
Filter membrane: PVDF、0.45uM

3. HPLC analysis conditions:
Detector: RI detector

Mobile phase: 0.03% NaN_3 in ddH2O
Flow rate: 0.6ml/min
Injection: 100 uL
Temperature: 35℃

The GPC separates samples based on the molecular weights and the substances with a higher molecular weight have a shorter retention time in the column, whereas the substances with a lower molecular weight have a longer retention time in the column because these substances can pass through the pores of the column, and the column can be used to analyze distribution of the molecular weight of the sample. The peak retention times of the calibration curve of the standards with a molecular weight between 20 and 1,600 kDa and the *Ganoderma lucidum* fermented liquid are shown in Fig. 3. The chromatograms and summary of the peak features of the *Ganoderma lucidum* fermented liquid are shown in Fig. 4. Peak 1 appeared at 11.5 minutes and, based on the retention time, the molecular weight of the polysaccharide of peak 1 is significantly higher than 1,600 kDa of the standard. Based on the calibration curve, it is estimated that the molecular weight may be up to 1,000 kDa and accounts for 58.9% of the total volume. Peak 2 appeared at 16.4 minutes and the molecular weight of the polysaccharide of said retention time is around 215 kDa and accounts for 41.1% of the total volume. The *Ganoderma lucidum* fermented liquid obtained by this preparation method mainly contains two groups of polysaccharides: 23~826 kDa

and 827~over 1,600 kDa based on the endpoint of the peak retention time. From past literatures, the molecular weights of the polysaccharides in *Ganoderma lucidum* fermented liquid, *Ganoderma lucidum* mycelium, and *Ganoderma lucidum* fruiting body are extremely high, usually between a few thousand and hundreds of thousands daltons and a molecular weight higher than 1,000 kDa is very rare. Therefore, one of the polysaccharide in the *Ganoderma lucidum* fermented liquid prepared by our company has a molecular weight higher than 16,00 kDa, indicating the substance of the *Ganoderma lucidum* fermented liquid has not been disclosed before and meets the novelty and non-obvious patentability requirements.

Present invention solved the difficulties encountered in prior art that *Ganoderma lucidum* fermented products cannot be used to improve alcoholic fatty liver, alcoholic liver disease, reduced hepatic antioxidant levels induced by alcohol, macrophage-related inflammatory diseases and intestinal inflammatory diseases. The results of the aforementioned examples proved that present invention provides a deep liquid fermented product of *Ganoderma lucidum*, including the *Ganoderma lucidum* mycelium and fermented liquid, which contains two groups of polysaccharides with a molecular weight of 23~826 kDa and 827~over 1,600 kDa and said fermented

product can 1) reduce liver function markers such as AST, ALT , serum triglyceride, cholesterol and blood glucose levels, indicating the *Ganoderma lucidum* fermented liquid can improve alcoholic liver disease. 2) reduce hepatic fat accumulation, for example, cholesterol and triglyceride, suggesting the *Ganoderma lucidum* fermented liquid can prevent and improve fatty liver. 3) increase the concentrations of antioxidants GST, GSH-Rd, GSH-Px and SOD and the activity of catalase, demonstrating the *Ganoderma lucidum* fermented liquid can improve the reduced liver antioxidative function induced by alcohol. 4) reduce secretion of TNF-α and IL-6, the inflammatory indicators, by macrophages. 5) reduce secretion of the pro-inflammatory marker IL-8 by intestinal tract, indicating the *Ganoderma lucidum* fermented liquid can improve the liver disease caused by alcohol and improve inflammatory diseases associated with macrophages and intestinal tract.

Liquid fermentation process for *Ganoderma lucidum*

In a preferred embodiment, the liquid fermentation process is to inoculate a fixed amount of *Ganoderma lucidum* (BCRC930182 and CGMCC12517) into a sterile culture medium containing combined carbon source 1~10%, combined nitrogen source 0.1~2%,

inorganic salts 0.01~0.3% and trace minerals 0.01~0.3% and incubate the culture for 3-15 days with stirring and ventilation to give a liquid fermented product. The liquid fermented product includes the mycelium and fermented liquid. The fermented product is then subjected to a solid-liquid separation process and said process involves addition of 3-15% diatomite to the fermented product and filtration to give a *Ganoderma lucidum* fermented liquid, wherein the combined carbon source includes but not limited to glucose, sucrose, fructose, maltose, maltodextrin, starch and the like; wherein the combined nitrogen source includes but not limited to animal proteins, for example, casein, whey protein, milk powder, vegetable protein, yeast and the like; wherein the solid-liquid separation process includes but not limited to addition of diatomite powder, filtration and centrifugation. The liquid fermentation process mentioned above is shown in Fig. 1. The method of fermentation is deep liquid fermentation for production of a viscous Ganoderma lucidum fermented liquid with a golden brown to brown color and a touch of mushroom flavor.

The foregoing detailed description of the invention and the specific examples are provided herein for the purpose of illustration only, and the invention is not limited to the preferred embodiments shown. It should be understood that any changes or modifications

within the spirit of the invention shall be included in the scope of present invention.

What is claimed is:

1. Use of a composition prepared in the manufacture of a health product or medicinal product that improves liver disease induced by alcohol and its use thereof, said composition comprises of the liquid fermented product of *Ganoderma lucidum*.
2. The use as recited in claim 1, wherein the liver disease refers to alcoholic liver disease, alcoholic fatty liver, reduced concentration of hepatic antioxidants caused by alcohol, and reduced activity of hepatic antioxidant enzymes caused by alcohol.
3. The use as recited in claim 2, wherein alcoholic liver disease refers to abnormal elevation of liver biochemical markers or abnormal liver function caused by alcohol.
4. The use as recited in claim 3, wherein abnormal liver biochemical markers refer to elevated serum AST or elevated serum ALT levels.
5. The use as recited in claim 3, wherein abnormal liver function refers to elevated fasting serum triglyceride levels, elevated cholesterol levels or elevated blood glucose levels.
6. The use as recited in claim 2, wherein alcoholic fatty liver comprises elevated hepatic triglyceride, cholesterol or total fat levels.
7. The use as recited in claim 2, wherein the hepatic antioxidant refers

to Glutathione.

8. The use as recited in claim 2, wherein the hepatic antioxidant enzymes include SOD (Superoxide dismutase), Catalase, GSH-Rd (GSH Reductase), GSH-Px (GSH Peroxidase) or GST (Glutathione S-transferase).

9. Use of a composition prepared in the manufacture of a health product or medicinal product that improves the inflammatory diseases associated with macrophages and its use thereof, said composition comprises of the liquid fermented product of *Ganoderma lucidum* obtained through liquid culture.

10. The use as recited in claim 9, wherein the inflammatory diseases associated with macrophages refers to diseases associated with increased secretion of TNF-α and IL-6 by macrophages, including hepatitis, nephritis, sepsis, arthritis or periodontitis.

11. Use of a composition prepared in the manufacture of a health product or medicinal product that improves intestinal inflammatory disorders and its use thereof, said composition comprises of the liquid fermented product of *Ganoderma lucidum* obtained through liquid culture.

12. The use as recited in claim 11, wherein the intestinal inflammatory disorders refer to disorders associated with increased secretion of IL-8 by intestinal cells, including Crohn's disease, ulcerative

colitis, or Rotavirus colitis.

13. The use as recited in claims 1, 9 and 11, wherein the features of the liquid fermented product of *Ganoderma lucidum* include the polysaccharides with a molecular weight between 23~826 kDa and polysaccharides with a molecular weight between 827 and over 1,600 kDa.

14. The use as recited in claims 1, 9 and 11, wherein said composition can further contain a flavoring substance or adjuvant and said flavoring substance is comprising of sugars, acids, fruit juices, condiments or water.

15. The use as recited in claims 1, 9 and 11, wherein said composition can be prepared in various dosage forms, including liquid nutrient supplements, liquors, beverages, powders, capsules, tablet, pills, granules, jellies, chewing gums, or other dosage forms that may be taken orally.

16. The use as recited in claim 14, wherein the sugars refer to substances that can provide sweetness, including but not limited to, sucrose, dextrose, fructose or sucrose; wherein the acids refer to any substance that can provide sourness, including but not limited to, citric acid, malic acid, tartaric acid or phosphoric acid; wherein the fruit juices refer to substances commonly used in modification of

beverage flavors, including but not limited to, apple juice, orange juice or grape juice; wherein the condiments refer to any substance that can change the odor, including but not limited to, synthetic flavors, vanilla seeds or mint extracts.