Ganoderma lucidum as Anti-Inflammatory Agent on The Level of Albumin and Globulin in Rat (Rattus Norvegicus) Rheumatoid Arthritis (RA) Model

by Eko Setio Wibowo

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S W Tiyah*, N I Ratnaningtyas, E S Wibowo, A Mumpuni, N Ekowati and A H Hikam

Faculty of Biology, Jenderal Soedirman University. Jl. Dr. Soeparno 63, Purwokerto, Banyumas 53122, Central Java, Indonesia. Tel./Fax. Tel.+62-281638794, Fax: +62-281-631700.

*Email: sestitiyah@gmail.com

Abstract. Inflammation is one of the immune system's responses to infection, irritation, also cell damage. Inflammation stimulates pro-inflammatory biomarkers. Albumin and globulin are included as inflammatory biomarkers, albumin is a negative acute phase protein (-APP), which will decrease, and globulin is a positive acute phase protein (+APP), which will increase due to inflammation. Ganoderma lucidum is a medicinal mushroom with anti-inflammatory potential that could increase the albumin level in blood due to inflammation. This research aimed to examine the effect of G. lucidum extract on the albumin and globulin level of inflamed white rats and determine the most effective dose of extract to be an anti-inflammatory agent. The study was conducted with a completely randomized design (CRD) consisting of 6 treatments of healthy control (HC), inflamed rats as negative control (C-), inflamed rats with Na-diclofenac administration (C+), and inflamed rats with G. lucidum extract administration with a dose of 250, 500, 750 mg/kg BW (T1, T2, and T3) with four replications each. The independent variable is the dose variant of G. lucidum (250, 500, 750 mg/kg BW), with the dependent variable being the change in albumin and globulin levels. The main parameters are albumin and globulin levels, and the support parameter is the GC-MS test. The data were processed using Analysis of Variance (ANOVA) at an error rate of 5%, followed by Duncan's analysis at 95% confidence level. The results show that mushroom G. lucidum extract administration with a dose of 250 mg/kg BW is the most effective dose to be ana dose of 250 mg/kg BW, which is the most effective anti-inflammatory agent.

Keywords: albumin, GC-MS, globulin, inflammation

1. Introduction

Cancer [1], tumor [2], kidney [3], and lung diseases [4] initially start with inflammation. Inflammation is described as the body's reaction due to physical trauma that can cause swelling, edema, redness, heat, pain, impaired function, and cell damage. The inflammatory response involves the activation of enzymes, the release of mediators, the extravasation of fluids, the migration of cells, tissue damage, and repair. It is frequently associated with pain and involves an increase in vascular permeability, an increase of protein denaturation, and membrane alterations [5].

When the body gets inflammatory stimulation, the phospholipids membrane activates macrophages for degradation using the PLA2 enzyme, which produces arachidonic acid metabolites. Arachidonic acid will activate the COX-1, COX-2, and LOX enzymes. COX-1 s in tromboxanthromboxane, COX-2 enzymes convert AA into prostaglandins that cause pain. LOX enzyme converts AA into leukotrienes (LTA4), chemotaxially activating mononuclear cells and Polymorphonuclear cells [6]. Leukotrienes LTA4 directs neutrophils to perform phagocytosis in wounds, but the number of neutrophils must be appropriate if it is still sustainable to damage cells and tissues [7]. Polymorphonuclear cells like neutrophils and eosinophils release free radicals, and excessive production of NO^- by the iNOS enzyme will cause oxidative stress that will inhibit the healing process of inflammation, a condition where the imbalance between free radicals and antioxidants can cause edema [8]. Mononuclear cells work to activate macrophages that will secrete proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 [9]. Nuclear factor-κB (NF-κB) triggers inflammatory cytokines IL-6 and IL-1β, stress response proteins including COX-2 and inducible nitric oxide synthase (iNOS). Enzymes of iNOS produce NO^- that can lead to multiple inflammation-related diseases [5].

Albumin and globulins are included in proteins synthesized in the liver. During the inflammatory phase, the albumin content plays a role in maintaining the osmotic balance of fluid inside and outside the blood cells so that blood volume is maintained [7]. The average half-life of albumin is 14-20 days [10], inflammation that causes albumin levels to drop caused by a shortened half-life so that albumin levels fall, and an increase in globulin can signal inflammation related to viruses, bacteria, or immune disorders [11]. Globulin is a broad class of plasma proteins with different functions and can be divided into three main fractions (α, β, and γ) [12]. Globulins play a role in the circulation of ions, hormones, and fatty acids. The average total serum protein level in rats is 6.0-8.2 g/dL, which consists of albumin 3.5-4.1 g/dL, and globulin levels of about 1.5-2.5 g/dL [13]. The albumin/globulin (A/G) ratio is a picture of changes in protein fractions in the blood. The A/G ratio can be used as an indication of immunity status. If the A/G ratio >1 indicates a good state of immunity, the other way around is if <1 indicates a bad immunity status (14).

The utilization of medical mushroom G. lucidum besides its benefits in the pharmaceutical field, can also be used as a nutraceutical (15). In addition, many products are produced in capsules, powders, creams, herbal tonics, and syrups (16). Modern pharmaceutical and nutritional research shows the potential of G. lucidum to have several physiological and therapeutic effects. Its benefits include immunomodulating activity (17), enhancing immune function (18), and antitumor activity (19).Prevent and treat several diseases such as bronchitis, asthma, hypercholesterolemia, hepatitis, hypertension, neurasthenia, leukopenia, and cancer, with various biological activities such as antitumor, immune regulation, hepatoprotection, and anti-inflammatory (5).

Research by (20) in-vitro study found terpenoids, steroids, phenols, and flavonoid compounds resulting from HPLC hydro-ethanol extract G. lucidum and proved that dose 100μg/ml could suppress the expression of proinflammatory cytokines, decreased NF-κB expression and suppress Nitric Oxide (NO-) the product of iNOS without giving toxicity effect. The bioactive compound G. lucidum can downregulate the expression of the COX-2 enzyme (21), iNOS, suppress excess free radical production (22), inactivation of NF-κB, and suppress macrophage cells which cause inflammatory symptoms and cell damage. The use of a dose of 600-1600 mg/kg.BW extract of G. lucidum, according to (23) using CCL4 induction that makes liver injury of rats showed a significant increase of A/G ratio compared with test animal Wistar rat.

Ganoderma lucidum is known as Immortal Mushroom because its benefits have been known for 4000 years as herbal medicine treats many diseases. Therefore, it is necessary to test the beneficial compound withanti-inflammatory potential. The study results are expected to provide scientific information regarding the content of *G. lucidum* compounds which are useful as anti-inflammatory agents in test animals in the using male Wistar rats (*Rattus norvegicus*) RA-model of albumin and globulin levels in the blood.

2. Materials and Methods

This research used white rats (*Rattus norvegicus*) as the experimental animal, came from the Wistar line, male, eight weeks, 200 grams weight, and healthy. Rats had been acclimatized for seven days before treatment. The 24 rats were divided into six groups; each group consisted of 4 rats.

2.1 G. lucidum Extraction

About 2000 grams *G. lucidum* powder was added into ethanol Pro-Analysis (PA) 10 L, homogenized, and tightly covered left for 24 hours. The results of macerate left for 24 hours are then filtered by the vacuum filtration method. Simplicia still present in the beaker glass is carried out 2nd re-maceration for 24 hours [(24)], and the filtration process is redefined. Separate ethanol solvent using a rotatory evaporator until viscous extract was obtained.

2.2 Test Solution Preparation

2.2.1 Carboxy Methyl Cellulose (CMC)

To make 0,5% CMC solution needed 0,7 g CMC powder and gradually added 140 mL of ddH2O until expanded [(25)]. Then about 0.0168 g of Na-diclofenac added.

2.2.2 Dimethyl Sulfoxide (DMSO)

Making a DMSO 5% solution of 420 mL requires 21 mL of 100% DMSO solution dissolved with ddH_2O homogenized until it reaches 420 mL.

2.2.3 Experimental animal treatments

A total of 0.1 mL of CFA was injected into the soles of the right mouse's feet subcutaneously [(26)] and left for seven days as an inflammation reaction. The 8th day starts *G. lucidum* extract administration orally using gastric sonde method for as long as 14 days [(27)] for T1, T2, and T3. Give aquades for HC and CMC+Na-diclofenac fot C+. The blood sample was taken through the orbital vena.

2.3 Albumin and Globulin level measurement

The collected blood sample ascentrifuged at a speed of 6000 rpm for 10 minutes. Thus, the blood plasma supernatant separated. Then blood plasma is used to determine albumin and globulin levels. Examination of total protein and albumin analysis done. Analysis of total protein levels was performed by Biuret reagent kit, and albumin levels were performed by the Bromcresol Green (BCG) method. Globulin levels are calculated after the data of total protein, and albumin levels are obtained [(28)]

2.4 GC-MS identification

The *G. lucidum* thick ethanol extract is analyzed using the GC-MS Agilent 6980N Network GC System with the Agilent 5973 inert MSD (70 eV direct inlet) detector. About two µl sample solution of *G. lucidum* ethanol extract was injected into GC MS, which has a J&W Scientific capillary column, HP-MS with a length of 30 mm, a diameter of 0.25 mm, and a thickness of 0.25 µm. Helium-carrying gas at a flow rate of 1 ml/min (constant) with a split ratio of 1:10. The programmed oven temperature is 50°C and isothermal for 5 minutes, the rate increases to 10°C/min, and the temperature is increased to 280°C for 15 minutes. The injector port temperature is 290°C, and the mass spectrometer interface is 230°C [(29)].

2.5 Ethical Approval

the experimental animals have received ethical approval from the health research ethics committee of the regional general hospital (RSUD) of Dr. Moerwadi, Solo, with number 515/IV/HREC/2021. The experimental animals were terminated using diethyl ether. The termination process was carried out by minimizing or eliminating the suffering of the animals based on the institutional animal care and use committee (IACUC). During the treatment of experimental animals, we refer to the 5F principle, which is free from fear and distress, free from discomfort, free from hunger and thirst, free from pain, injury, and disease, and also free to express natural behavior.

2.6 Data Analysis

The data then were processed using an Analysis of Variance (ANOVA) at an error rate of 5%, followed by Duncan's analysis at a 95% confidence level using SPSS software ver. 25.0.

3. Result and Discussion

The blood sample that has been taken then goes through the stages of albumin and globulin levels analysis. Using a spectrophotometer to find absorbance value, the albumin and globulin levels could be determined using the albumin globulin formula. The existing data is then processed using the SPSS application.

Table 1. Average Value of Albumin and Globulin Levels in Rats with CFA Induction

Treatment	Average Value of Albumin & Globulin Levels (g/dL)	
	Albumin	Globulin
HC	4.12 ± 0.05^{c}	1.27 ± 0.37^{a}
C+	3.61 ± 0.34^{b}	1.27 ± 0.25^{a}
C-	2.87 ± 0.11^{a}	1.52 ± 0.27^{a}
T1	4.21 ± 0.13^{c}	1.32 ± 0.81^{a}
T2	4.26 ± 0.19^{c}	1.17 ± 0.18^{a}
T3	3.89 ± 0.42^{bc}	1.17 ± 0.52^{a}

Information: Numbers followed by the same letter are not significantly different at the P<0,05 level of significance. HC = Healthy Control (aquadest), C+ = Positive Control (Na-diclofenac), C- = Negative Control (suffer inflamation), T1 = *G. lucidum* 250 mg/kg.BW, T2 = *G. lucidum* 500 mg/kg.BW, T3 = *G. lucidum* 750 mg/kg.BW

Table 2. Albumin/Globulin Ratio and Immunity Status

Treatment	A/G Ratio (g/dL)	Immunity Status
HC	3.24	Good
C-	1.89	Good
C+	2.84	Good
T1	3.19	Good
T2	3.64	Good
T3	3.32	Good

Note: Good immune status = A/G ratio ≥ 1

The albumin level of rats showed a significant (p<0.0.5) positive effect of *G. lucidum* administration, while the globulin level didn't show a significant reaction (p>0.05). Treatment T1, T2, T3, and HC significantly differed with C+ and C-. Albumin levels of the C- treatment had levels below normal mice, while other treatments were still within the range of normal albumin levels in healthy mice (Tabel 1). The research of [30] showed that inflammation causes the amount of albumin levels to decrease due to reduced albumin synthesis. [12] stated that albumin is a negative phase protein (-APP), where the amount will decrease when inflammation occurs.

The C- treatment has low albumin levels because it did not get treatment or administration of *G. lucidum* extract. Low albumin levels during inflammation are caused by an increase in the cytokine IL-6, which attracts albumin from intravascularly to the liver and decreases albumin synthesis [31]. [32], stated that testing the administration of 500 µg/ml hydro alcohol extract of *G. lucidum* in-vitro can inhibit albumin denaturation by 77% at the time of inflammation. According to the T1 treatment, T2 and T3 had higher average albumin levels than C-. The administration of Na-diclofenac in the C+ treatment can increase albumin levels because Na-diclofenac is nonsteroidal anti-inflammatory drug (NSAIDs) that can bind albumin to the blood when inflammation occurs [33]. [34] Also, doses of 100 mg/kg BW were already effective in increasing albumin levels in CCL_4-induced rat liver. Research by [35] supports study's results by mentioning the administration of ethanol extract of *G. lucidum* at a dose of 100-800 mg/kg.BW does not have a toxic effect on the liver, so the synthesis of albumin and globulin levels remains normal.

Globulin levels will drop if they experience malnutrition or congenital immune deficiency [36] or if the test animal has liver or kidney problems. Conversely, globulin levels will rise if there is inflammation or infection and the nutrients are eaten [37]. The results of the average globulin content showed that rats given inflammatory treatment showed relatively higher globulin levels compared to other treatments, although the results of the increase were not significant. This result corresponds to [37], where inflammation makes globulin levels rise despite an insignificant increase. This result is different from the study of [38], where a dose of 300 mg/kg BW *G. lucidum* can reduce globulin levels in Ochratoxin A (OTA) induced rats, making globulin levels ret to the normal range. However, the *G. lucidum* ethanol extract dose showed that globulin levels of T1, T2, and T3 were lower than C-.

Table 2 shows that all treatments of HC, C+, C-, T1, T2, and T3 have good immunity status (>1) [14]. The T2 treatment has the highest A/G ratio, and C- has the lowest A/G ratio. This shows that CFA jection treatment in test animals can reduce the A/G ratio in terms of C- and a higher A/G ratio due to the administration of *G. lucidum* ethanol extract in the application of T1, T2, and T3 doses. According to [39] A/G ratio between 1.1 and 2.5 is considered normal. The results showed that the A/G ratio was relatively high (>1.1 or >2.5). The A/G ratio will be high if albumin levels are high and globulin levels are normal or if normal albumin levels and globulin levels are low.

Measurement of total protein, albumin, and globulin can help diagnose diseases of the kidneys or liver. When protein intake and/or A/G ratio is less, amino acids will be synthesized into albumin. The amount of albumin below normal levels indicates poor liver function [14]. The research of [26], found that *G. lucidum* possesses a hepatoprotective effect on the rat induced CCL_4 by changing MAT expression and significantly increasing the A/G ratio in doses of 600 and 1600 mg/kg BW.

When inflammation occurs, proinflammatory cytokines, especially IL-6, will be activated. The production of IL-6 will withdraw the circulation of albumin from the blood circulation. Inflammation will also make albumin synthesis in the liver stop and trigger the liver to produce CRP, increasing globulin synthesis that can alter the protein profile of the liver. Oxidative stress can also cause liver cell damage, affecting the liver's protein profile. The synthesis of NO by the enzyme iNOS meets free radical O_2^- which will form $ONOO^-$ which is unstable and make stress oxidative. *G. lucidum* also contains antioxidant compounds to neutralize oxidative stress, such as triterpenoid compounds. Triterpenoids will donate H^+ ions to stabil free radicals. The content of triterpenoid compounds in *G. lucidum* can weaken the transcription of the NF-κB proinflammatory gene and decrease the activation of IL-6. The GC-MS test using thick ethanol extract of *G. lucidum* (Appendix 6) showed ten dominant compounds (Table 3). The dominant compounds of *G. lucidum* extraction are grouped into six groups. There are three compounds that belong to the fatty acid group, compounds including the acetic group, 2 compounds including the terpenoid group, then three compounds each including the ketone group, amino acids, and peptides.

Fatty acid group compounds include (2-fluoro-benzyl)-3,4,5-trimethoxy-benzamide; 6-OXO-1,6-Dihydropyridine-3-carboxylic acid (2-methoxy-dibenzofuran-3-YL)-amide; and 6-(N,N-Dimethylamino)-4-cyano-1,3-dimethyl-4-hexen-3-ol. The acet acid groups detected were N-(4-Cyanomethyl-phenyl)-2-(3,5-dimethyl-phenoxy)-acetamide and 8-acetoxy-6-benzenesulfonyl-2-thia-6-aza-adamantan-4-yl esteer. Compound Cholesta-5,20,24-trien-3-ol, (3beta.) -(CAS) and 9,19-Cycloanost-23-en-3-ol-methoxy-,acetate,(3beta.,23E)- (CAS) belong to the terpenoid group. The compound Tungsten, tris (eta-4-3-methyl-3-YL)-amide belongs to the ketone group. There is also a group of amino acid compounds Methyl N-4-bromobrnzoyl-l-valyl-2-methylalanyl-2-methylalaninate. Last, peptide group compounds are Trisulfide, dipropyl (CAS). The fatty acid group has the largest concentration of 18.66%, with the dominant compound 6-(N,N-Dimethylamino)-4-cyano-1,3-dimethyl-4-hexen-3-ol has a concentration of 13.91%, and the group with the lowest concentration is ketone, with the compound Tungsten, tris (eta-4-3-methyl-3-YL)-amide has a concentration of 8.92%.

Table 3. GC-MS Result of *G. lucidum* Ethanol Extract Compounds

No.	Time Retention	Compound Name	Concentration (%)	Compound Group
1.	3.575	N-(2-Fluoro-benzyl)-3,4,5-trimethoxy-benzamide	8.79	Fatty acid
2.	4.315	N-(4-Cyanomethyl-phenyl)-2-(3,5-dimethyl-phenoxy)-acetamide	9.76	Acetic acid
3.	7.24	Cholesta-5,20,24-trien-3-ol, (3beta.)-(CAS)	8.79	Terpenoid
4.	11.679	6-OXO-1,6-Dihydropyridine-3-carboxylic acid (2-methoxy-dibenzofuran-3-YL)-amide	11.31	Fatty acid
5.	13.34	Tungsten, tris (eta-4-3-methyl-3-YL)-amide	8.92	Ketone
6.	13.485	Methyl N-4-bromobrnzoyl-l-valyl-2-methylalanyl-2-methylalaninate	9.26	Amino acid
7.	13.775	Acetic acid, 8-acetoxy-6-benzenesulfonyl-2-thia-6-aza-adamantan-4-yl esteer	8.9	Acetic acid
8.	15.397	9,19-Cycloanost-23-en-3-ol-methoxy-acetate,(3beta.,23E)- (CAS)	11	Terpenoid
9.	18.256	6-(N,N-Dimethylamino)-4-cyano-1,3-dimethyl-4-hexen-3-ol	13.91	Fatty acid
10.	21.28	Trisulfide, dipropyl (CAS)	9.36	Peptide

The group of fatty acid compounds, terpenoids, acetic acids, ketones, amino acids, and peptides identified in the GC-MS ethanol extract *of G. lucidum* hasa contributes to inflammatory relief. [40] conducted a GC-MS test of *G. lucidum* with petroleum ether solvents found groups of compounds that include fatty acid compounds, including lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid, stearic acid, docosanoic acid, and lignoceric acid. According to [41], fatty acids produced from gano oil, namely Oleamide, Hexadecanamide, and Octadecadienoic acid, with doses of 10, 25, and 50 mg/kg could inhibit paw edema. Oleamide inhibits pro-inflammatory mediators such as NO^- and PGE2, as well as expression of iNOS and COX2 and the anti-inflammatory effect, is through inhibition of NF-κB.

Terpenoid group compounds are proven to work as antioxidants and anti-inflammatories. Triterpenoids are derivatives of terpenoid compounds that can reduce pro-inflammatory cytokines, inhibit 10% COX-2 enzyme, and suppress iNOS at protein and mRNA levels. Attenuated by NF-κB, which are proinflammatory, pro-adhesion, and pro-oxidant gene transcription. Antioxidant activity and the ability to capture free radicals of triterpene compounds are related to the mechanism of hepatoprotection. Triterpenes provide antioxidant effects by donors H^+ ions to $ONOO^-$ and stabilize free radicals and restore lipid peroxidation [42].

4. Conclusion

Administration of *G. lucidum* ethanol extract with variant doses has a significant effect on albumin levels, but not on globulin levels in the blood of inflamed rats. *G. lucidum* ethanol extract doses of 250 mg/kg BW were effective to maintain the A/G ratio in balance with increased albumin levels.

5. Acknowledgments

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