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PRE-CLINICAL EVALUATION OF LOWERING ATHEROSCLEROSIS RISK FACTORS IN HYPERCHOLESTEROLEMIC RATS AND THE IMMUNOMODULATING ACTION OF *GANODERMA LUCIDUM* AND *CHRYSANTHEMUM INDICUM* LINN

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Abstract

*This research aimed to address cardiovascular disease, specifically atherosclerosis, by using *Ganoderma lucidum* and *Chrysanthemum indicum*, and evaluate their immunomodulating action. Beta-glucans from *G. lucidum* and extract from flowers of *C. indicum* were used. Toxicological analysis was done. Biochemical factors in *Rattus albus* like serum total cholesterol, triacylglycerol (TAG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) were assayed. The rats were fed on high fat diet and induced with cholesterol, then treated with the different treatments. Immunomodulation was evaluated by measuring the immunoglobulins A, G and M on *Escherichia coli*-infected rats before and after*

treatment. For proliferation of lymphocytes, whole blood was collected on carrageenan inflammation-induced rats before and after treatment and determined through flow cytometry. Results show a decrease in the TAG ($t=1.329$, sig. 0.211), TC ($t=4.956$, sig. <0.0001), HDL-C ($t=0.486$, sig. 0.636) and LDL-C ($t=0.819$, sig. 0.429) after treatment with the extracts but only the decrease in total cholesterol (TC) was significant at α 0.05. Paired t -test show a significant decrease in IgM ($t=2.969$, sig. 0.013) and IgG ($t=3.865$, sig. 0.003) while no significant decrease in IgA ($t=1.683$, sig. 0.130). Comparison of the treatments using ANOVA showed significant differences between treatments in IgA ($F(3,8)=8.458$, $p=0.007$.) and IgG ($F(3,8)=174.115$, sig. <0.0001) but not significant in IgM ($F(3,8)=3.998$, $p=.148$). There was also significant increase in the number of lymphocytes on *R. albus* ($t=2.659$, sig. 0.019) but ANCOVA showed no significant differences in the different treatments ($F=0.805$, sig. 0.530) suggesting that all treatments were comparable in their effects in proliferating lymphocytes. It can be concluded that *G. lucidum* beta glucans and *C. indicum* flower extracts are safe and effective either as single dose or in combination in lowering atherosclerosis risk factors and in immunomodulation.

Keywords

Cardiovascular Disease, Atherosclerosis, Immunoglobulins, Lymphocytes, Ganoderma Lucidum, Chrysanthemum Indicum

1. Introduction

Cardiovascular disease (CVD) is listed by the World Health Organization (WHO, 2015) as the leading cause of mortality and morbidity around the globe. At present, WHO reported that deaths due to CVD is 13.2% of the total population worldwide and the primary contributing factor for CVD is atherosclerosis, a chronic and systemic process which is characterized by several risk factors. Several drugs have been proven to lower these risk factors, but problems on unwanted side effects of these synthetic drugs led to the search for new viable alternatives. Undeniably, the globally growing public health burden of CVD required a cheaper alternative treatment which could be gained from various natural sources.

A wild strain of *Ganoderma lucidum* (GL) is present in abundance in Mt. Palali, Quezon, Nueva Vizcaya, Philippines. This is a very popular mushroom which catches the attention of research due to its wide spectrum of biological activities (Chen et al., 2002; Wani et al., 2010;

Khatun et al., 2012). Nowadays, several health supplements of GL are marketed in the form of coffee, tea, capsules and tablets. However, they do not come in cheap prices. While it is true that GL is active, its processing into a final product is important because people who take in the finished products may not really be benefitting from it. Review from literatures shows that the active components of the mushroom are found in their fruiting bodies and spores. Since the fruiting body of the mushroom is woody and not considered as food, supplements are usually made from processed basiodiocarps and spores which are not easily broken down even by stomach acids to liberate the essential secondary metabolites. In China, they have a unique way of cracking the bioactive components from this mushroom. However, their method is a trade secret. Today, several studies show that these spores from commercial supplements pass through the gut and found whole in stools. With this problem on bioavailability of active components of GL, an innovation on a better way to harness these chemicals that address CVD is a must.

Alternatively, chrysanthemum is an ornamental plant which is widely cultivated for agricultural livelihood in the locality of Nueva Vizcaya. Notably, its flowers are one of the traditional herbs being used by the Chinese, Japanese and Koreans to prevent and cure diseases (Xin and Jing, 2012; Jeong et al., 2013). *Chrysanthemum indicum* Linn. (CIL) or manzanilla is a wild herb and has a long history of use as a traditional drug for the cure of respiratory diseases, disorders of hypertension and inflammation. Several studies had proven the ability of chrysanthemum in treating various immune-related diseases. At present, added investigations such as the hypotensive activity, antimicrobial, antioxidant, tonic sedative, hepatoprotective effect and promotion of myocardial blood circulation are reported (Eddouks and Zeggwagh, 2014; Lograda et al., 2013). Furthermore Shen et al., (2004) found that chemical composition of this plant was shown to be depended on microclimatic occurrences and soil conditions where it is grown.

Ganoderma lucidum is a very popular mushroom known as *reishi* or *mannentake* and “Mushroom of Immortality” to the Japanese, *Ling Zhi* to the Chinese and *Kabuteng Kahoy* in our locality. Nowadays, GL is becoming an ideal constituent of a healthy diet due to its great nutritional and health beneficial qualities. Numerous studies concerning the extensive herbal properties and actions of GL are continuously reported including its antimicrobial, anticancer, hepatoprotective, antioxidatives, anti-allergic, kidney tonic, nerve tonic (Amin et al., 2012; Khatun et al., 2012) and its ability to lower serum cholesterol concentration and blood pressure

(Wani et al., 2010). GL contain about 400 diverse bioactive compounds which include polysaccharides, sterols, fatty acids, amino acids, triterpenoids, steroids, peptides and proteins, vitamins, dietary fibers, etc. which attributed to its medicinal effects (Amin et al., 2012; Khatun et al., 2012). Mushroom containing biologically lively polysaccharides mostly included in the group of β -glucans. β -Glucans increase the immune defense of the host by stimulating the complement system, macrophages are being enhanced and the functions of the natural killer cells. It has been known that β -glucan is the most powerful immune stimulator known and a powerful antagonist against tumors for both benign and malignant, it is also known for its capacity to lower the cholesterol and triglyceride levels, stabilize levels of blood sugar, heals and rejuvenate skin and it has other benefits.. β -Glucan activates the immune response through the immune cells, called macrophages, showing various therapeutic effects. In recent years β -glucan has been in a focus of intensive research, primarily because it is a safe and very potent biological response modifier (Akramienė et al., 2007).

Chrysanthemum indicum Linn. is a member of the Asteraceae family. Chrysanthemum was nurtured as one of the noble plants in ancient China and is highly valued for its medicinal properties. Chrysanthemum has been used in several Asian countries, such as China, Japan and Korea as a traditional herbal medicine. It has also been used as a traditional drink in Chinese legends for rejuvenation and prolonging life and presently known as Chrysanthemum tea. It is an herbaceous perennial herb that can spread densely and grow between 30-60 centimeters in height. It favors warm climate. The stem of this plant is green when young and become brown as it age. The leaves are serrated ovate-shaped, thin and propagates 4-6 centimeters long. The flower heads are yellow and have a flat-top flower cluster. The flowers continue to bloom throughout the year but it happens mostly in cold season.

This plant is traditionally reported for its analgesic, anti-inflammatory and antipyretic properties (Cheng et al., 2005; Pradhan et al., 2011). Moreover, several studies had proven the ability of Chrysanthemum in treating various immune-related diseases. They have recognized its biological features such as hepatoprotective (Jeong et al., 2013), anti-bacterial and anti-fungal (Kommidi et al. 2014), cardiovascular protection (Lii et al., 2010), anti-inflammatory (Wu et al., 2013) and anti-aging (Yagi et al., 2012). This herbal tea also used in treating pimples and acne and also effective in detoxifying the liver and lowering the cholesterol. There are some disease that chrysanthemum tea can help with its treatment like the coronary artery disease, blocked

arteries and varicose veins. It has a stimulating property that can help in alerting the senses and rejuvenating the brain that can stimulate all your senses in alert as well as calming down the nerves. Likewise, chrysanthemum has high amounts of β - carotene which are present in the yellow part and the fruit. The β -carotene is converted in Vitamin A in the liver. This kind of Vitamin A is helpful in treating skin problems and increasing the immunity power (Trey, 2014). Moreover, it is well known to be an effective herbal beverage which has flavonoid, a main active component of the flower that has important role in exerting anti-inflammatory effects and antioxidant activities (Kommidi et al., 2014).

Dietary recommendations are natural, well known and cost-effective way of eradicating diseases. Reports of *G. lucidum*'s medicinal properties such as cholesterol serum reducer, antiviral, immune enhancer, anti-tumor and anticancer considered it as important part of the human diet (Wani et al., 2010; Khatun et al., 2012). Hence, with the optimized method of mass production of GL set by Magday, Dulay and Bungihan (2014) and innovation on a better way to harness active components from the spores and fruiting bodies of the mushroom with the supplementary effect of the flowers of CIL, bioavailability and effectiveness of the samples as cost-effective dietary supplements for the people who are suffering from cardiovascular disease (CVD) will be affordable. Likewise, the mass production of the wild strain of GL and the mass propagation of CIL in our locality will provide livelihood for our low income families. Furthermore, the evaluation of the biochemical activity of the wild strain of GL and CIL in lowering the risk factors of atherosclerosis will serve as a pre-clinical trial leading to more advanced research endeavors such as the isolation and utilization of the active components for clinical development of drugs which will be used in pharmacological treatment of CVD.

With the increasing interest of today's populace towards natural products, GL and CIL could be a promising source of bioactive components that could be used as an alternative natural medicine to unlock CVD complications. This study aimed to evaluate the effectiveness of *Ganoderma lucidum* and *Chrysanthemum indicum* Linn. in lowering the risk factors of atherosclerosis and in increasing immunity. The specific objectives of the study were as follows.

- To extract beta-glucans from *G. lucidum* fruiting bodies
- To extract the phytochemicals from *C. indicum* flowers
- To evaluate toxicity of *G. lucidum* and *C. indicum* singly and in combination on rats

- To evaluate the *G. lucidum* and *C. indicum* extracts by testing in rats' serum the following: (a) low density lipoprotein cholesterol (LDL-C), (b) High density lipoprotein cholesterol (HDL-C), (c) Total cholesterol (TC) and (d) Triacylglycerol (TAG)
- To evaluate the immunomodulating effects of *G. lucidum* and *C. indicum* by testing in rats through assessment of (a) decrease of lymphocytes, and (b) increase in immunoglobulin levels.

The study is significant in the generation of enhancing the immune system in fighting against invaders and the boosting effect in the immunity derived from GL and components of CIL allowing researchers to be well acquainted with the immunomodulating action of the samples in the decrease of proliferation of lymphocytes and increase in IgG, IgM and IgA as a measure of enhanced immune system.

The mechanisms by which GL and CIL may affect the immunomodulating functions of immune system through decrease in proliferation of lymphocytes and immunoglobulins A, M and G are unknown. With this study scientific methods are being endorsed to everybody, which may lead to having low-cost alternative immune booster without compromising the quality. Moreover, a pre-clinical testing of the supplementary medicinal effect of the flowers of CIL along with the ability of GL to reduce serum cholesterol is needed to evaluate the effectiveness of these extracts in lowering the risk factors of atherosclerosis.

2. Methodology

2.1 Research Design and Research Locale

The study used factorial experimental design where different factors/variables were measured. It used experimental design where control groups (negative and positive controls) and treatment groups were measured against these factors and their effects were compared. The effect of *G. lucidum* alone, *C. indicum* alone, and a combination of both against negative and positive controls were compared. The study was conducted at the laboratory of the Center for Natural Sciences, Saint Mary's University, Bayombong Nueva Vizcaya, Philippines. The study composed of two phases.

2.2 Collection and Preparation of *Chrysanthemum indicum* Linn. Flowers

Plant samples were collected from the flower plantation found in Kayapa, Nueva Vizcaya and from a backyard in Bayombong, Nueva Vizcaya. Healthy matured flowers were placed in

clean plastic bags and transported to the laboratory of Saint Mary's University, Bayombong, Nueva Vizcaya. Flowers were air-dried and powdered using a laboratory blender. The flower samples were soaked in 95% ethanol. After filtration, the extracts were placed on a water bath at 45 °C until they became syrupy. The crude extracts were weighed and stored inside the refrigerator until ready for use.

2.3 Mass Production and Extraction of *G. lucidum*

With the optimized conditions set by Magday and Bungihan (2014), GL mycelia were introduced into mushroom culture beds previously set-up for mass production. Spores were collected using paper caps set around the basidiocarps. After sporulation is finished, fruiting bodies were collected and processed for the isolation of secondary metabolites. Ground form of *G. lucidum* fruiting bodies and spores were extracted with boiling water for 3 h. The pH was adjusted to 10 through the addition of saturated solution of sodium hydroxide. The water extracts were processed through several purification steps to remove other substances. Proteins were removed by precipitation with 20% of trichloro-acetic acid and separated by centrifugation. To obtain brown crude glucans, polysaccharides were precipitated from the supernatants by the addition of 2:1 ratio (v/v) of ethanol. Concentrated sodium chloride were added to favor precipitation. The beta-glucans were be dried, weighed and stored until ready for use.

2.4 Toxicological Testing of *G. lucidum* and *C. indicum* on *Rattus albus*

Toxicological screening was adopted from Guevara (2004). It used the approximate lethal dose (ALD) method and the basic pharmacological-toxicological effects.

2.4.1 Determination of the Approximate Lethal Dose (ALD) by Single Dose Method

The maximum volume administered to the experimental animals did not exceed one mL of the test drugs and the control drugs (GL and CI and Normal saline solution). To determine the dose levels by logarithmic method at 0.6 intervals, a convenient starting dose of 10 mg/kg body weight was chosen. To determine the second dose, the log of 10 was taken and 0.6 was added. To determine the third dose, log of 39.80 was taken and 0.6 was added. The next higher doses were similarly computed. The table shows the summary of the dose levels.

Table 1: Dose Levels by Logarithmic Method

At 0.6 intervals	Calculated dose in mg/kg	Dose mg/kg BW (rounded to the nearest whole number)
1.0	10.0	10
1.6	39.8	40
2.2	158.5	160
2.8	631.0	630

The test drugs (extracts) were administered intraperitoneally to the experimental animals at an arbitrary initial dose of 10 mg of the test drug per kg body weight (BW) of the animal, expressed as 10 mg/kg BW. The dose levels were increased logarithmically at 0.6 log intervals. Then the weight of the plant extract needed for the tests was computed based on the calculated dose levels and the body weight of the experimental animals. The calculated amount was administered until two consecutive doses were obtained in which the lower dose level did not produce any immediate toxic effects whereas the next higher dose was observed to be lethal to the experimental animals. To another group of experimental animals, normal saline solution (NSS) or the vehicle used to suspend the test drug was administered. The number of deaths and the time of deaths per group within 3 days after drug administration were recorded. The dosage range for these observations was noted.

2.4.2 Basic Pharmacological – Toxicological Effects

Observation on the experimental animals for the basic pharmacological – toxicological effects was adopted from Guevara (2004). The different effects of the test drug to the experimental animals were conducted for 48 to 72 hours post treatment. The different categories of the basic pharmacological – toxicological effects were central nervous system (CNS) depression, CNS stimulation, eye observation, ear observation, general observation and subjective tests.

2.5 Assay and Measurement of Biochemical Factors in *Rattus albus*

Rats of the same age group were purchased from a breeder to ensure the uniformity of the age and at least the differences in body weights were only a small range. Healthy rats (*Rattus albus*) weighing from 150-250 g were brought to the laboratory for acclimatization. The animals were fed with high fat diet of beef fat and cholesterol added to their normal diet for 30 days to induce hypercholesterolemia (Zhukova et al., 2014). Rats were randomized into four groups: group I (treatment of ganoderma), group II (treatment of chrysanthemum), group III (treatment

with both ganoderma and chrysanthemum (1:1) and group IV (treatment with Simvastatin) each consisting of 3 replicates. The method used is a modification of Setorki et al. (2010). Baseline blood samples were drawn from the tail of the rats before they were given treatments. Test animals with high fasting cholesterol serum levels were selected for the assay. Isolated bioactive compounds from *G. lucidum* and *C. indicum* were administered to the experimental group through oral gavage for 2 weeks. After the treatment, blood samples of the rats were centrifuged at 3500 rpm for 20 minutes to obtain serum used as biomarkers. Serum total cholesterol (TC), triacylglycerol (TAG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C) were measured. Assay for the biochemical markers was done following the protocols for Triacylglyceride Quantification Assay Kit (ab65336, abcam, USA) and Cholesterol Assay Kit (ab65390, abcam, USA). Before and after treatment data were recorded in tables for data analyses.

2.6 Immunoglobulin Test Measurement

Another set of rats were used in this phase. There were five treatment groups, each containing 5 replicates each- Treatment 1: Negative Control (NSS), Treatment 2: Combination of GL and CI, Treatment 3: *C. indicum* only, Treatment 4: *G. lucidum* only, and Treatment 5: Positive control (Vitamin C). The levels of immunoglobulins were tested using the ELISA.

Inflammation was induced by injecting *E. coli* to the rats' subcutaneous tissue. The amount of bacteria injected depended on the body weight of the rats. After 24 hours of infection, animals received NSS only (I), both CI compounds and GI β -glucan (II), CIL extract (III), GL β -glucan (IV), and Vitamin C only (V), throughout the whole experimental period. A time interval of 6 hours between the administration treatments were given to avoid disturbances to the absorption of each substance. Blood from the rats were withdrawn from their tails. The whole blood was centrifuged at 4,700 rpm for 10 minutes to separate the serum. Diluted serum samples were placed in 96-well plates coated with antigens and incubated for several hours with primary antibodies then washed. Then the secondary antibodies were added and incubated for several hours and washed again. Other necessary protocols were followed from the Ig test kits (Rat IgG, IgM and IgA ELISA Kit, ICL, Inc., USA) purchased. The samples were read in an ELISA reader at 450 nm. Immunoglobulin G, Immunoglobulin M and Immunoglobulin A were measured spectrophotometrically using ELISA in all groups of rats to compare the results.

2.7 Lymphocyte Test Measurement

Lymphocytes were measured using flow cytometer. Baseline measurement was done before inflammation. Then, inflammation was induced on healthy rats by injecting carrageenan on rat's paw to induce edema. After observed inflammation, the rats were administered with the different treatments every 6 hours for 24 hours. The effects of the test drugs were then evaluated. Whole blood collected in EDTA microtainer were subjected to a flow cytometer machine and has the same principle as complete blood count. The results obtained were expressed in percentage corresponding results of the concentration of different leukocytes. Proliferation of lymphocytes was evaluated against Vitamin C as positive control.

2.8 Data Analysis

SPSS software v.21 was used in statistical analysis. Values were statistically evaluated using analysis of covariance (ANCOVA) or analysis of variance (ANOVA) for significant differences of the different treatment groups. Paired T-test was used for the treatment of data on the decrease of cholesterol levels in rats, decrease in immunoglobulins and increase in lymphocytes. Different treatments were used to test the immunomodulation effect of CIL and GL on samples. The data obtained were placed in tables and the means were obtained. The mean obtained from ELISA readings and flow cytometry readings listed from the tables were analyzed by one-way analysis of variances (ANOVA) or ANCOVA if the results show significant results at α 0.05. The graph of the levels of cholesterol, immunoglobulins and levels of lymphocytes were drafted using the Microsoft Excel.

3. Results and Discussion

3.1 Toxicological Testing of *G. lucidum* and *C. indicum* on *Rattus albus*

The experimental animals were fed through oral gavage with logarithmic doses of the beta-glucans alone, chrysanthemum extract alone and combination of both. The negative control was fed with NSS. Results show that all treatments were non-toxic and non-lethal with average lethal dose greater than 1500 mg/kg body weight (ALD >1500 mg/kg BW) even in extended observation period of 72 hours. There was no significant increase or decrease in body weight and body temperature and heart rates were normal. For the basic pharmacological-toxicological effects, the observation guide was derived from Guevara (2004), and was done in 24 hours and up to 72 hours. It was observed that there was no CNS depression effect except for mild

analgesia, suggesting that the extracts have analgesic properties. There was no marked CNS stimulation observed. Eye observation was normal for all set-ups. For ear observation, only mild hyperaemia was noted. This might be due to the increased blood flow in the capillaries in the rat's ears. Though the condition was only mild and had returned to normal after 24 hours. For general observations and subjective tests, all were observed normal. From both ALD and basic pharmacological tests, it can be concluded that *G. lucidum* beta-glucans and *C. indicum* flowers are safe and non-toxic when used alone or in combination.

3.2 Assay and Measurement of Biochemical Factors in *R. albus*

In recent years β -glucan has been in a focus of intensive research, primarily because it is a safe and very potent biological response modifier (Akramienė et al, 2007). Figure 1 shows the triglyceride values of rat blood sera before and after treatment of the drug and extracts. Values of TAG before treatment were the triglyceride levels of rats induced for hypercholesterolemia. Values of TAG after were taken after they were administered with the different treatments. It can be gleaned from the values that there was a decrease in serum triglyceride levels in *R. albus*. It is noteworthy that the combination of ganoderma and chrysanthemum showed very large decrease in triglyceride levels when compared to other treatments. However, t-test showed that the overall decrease in all treatments were not significant ($t=1.329$, sig. 0.211). Also, comparison of the triglyceride levels using ANOVA showed that there are no significant differences between treatments ($F=2.305$, sig. 0.154).

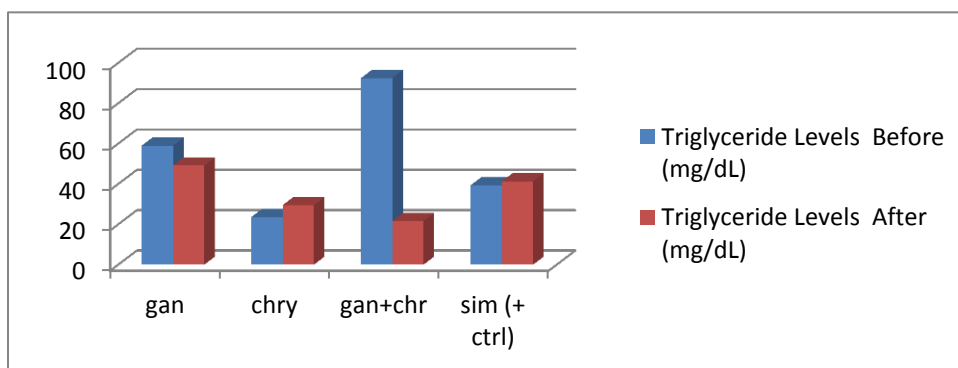


Figure 1: Serum triglyceride levels in *R. albus* before and after treatment

Analysis using t-test showed that the significant decrease was only in the total cholesterol (TC) level where all treatments consistently showed decrease ($t=1.329$, sig. <0.0001) at $\alpha=0.05$. HDL-C and LDL-C showed no significant decrease. Figure 2 shows the serum total cholesterol levels in *R. albus* before and after treatment while Figures 3 and 4 shows the decrease in HDL-C and LDL-C for all treatments.

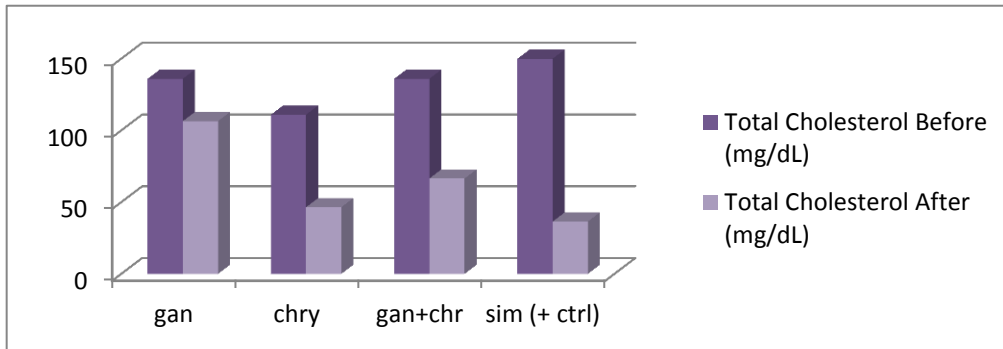


Figure 2: Serum total cholesterol levels in *R. albus* before and after treatment

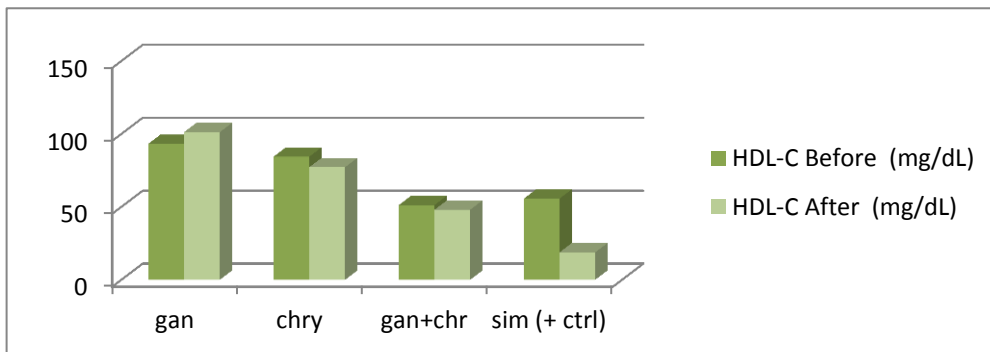


Figure 3: Serum high density lipoprotein-cholesterol (HDL-C) levels in *R. albus* before and after treatment

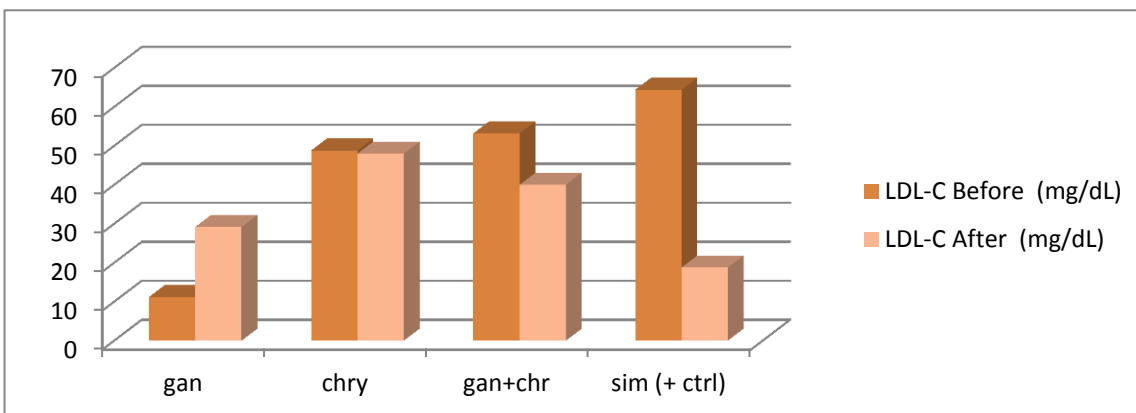


Figure 4: Serum low density lipoprotein-cholesterol (LDL-C) levels in *R. albus* before and after treatment

The rats were of average age of 15 months which is equivalent to 40 years in human years. The normal values for rat cholesterol ranges from 50-250 mg/dL (UPSVM, 2002). The elevated levels of triglycerides in the blood increases the risk of heart disease. There is a need to understand the interplay of cholesterol with that of the biochemical risk factors like HDL and LDL. An increased HDL and decrease in LDL would reduce the risk for cardiovascular disease. Cholesterol alone cannot be used as a marker for CVD. In this scenario, the marked decrease in triglyceride levels, in total cholesterol, an insignificant decrease of HDL and a larger decrease in LDL can support the claim for the benefit of combining both ganoderma and chrysanthemum to address cardiovascular disease, particularly atherosclerosis risk.

3.3 Immunoglobulin Test Measurement

3.3.1 Immunoglobulin G

Before the rats were induced with infection, their baseline IgG levels were determined. Then, *E. coli* were injected and after 24 hours of infection, the IgG levels were determined by ELISA to check for the immune response of the rats as shown by the increase in their IgG levels. Then, introduction of the different treatments was done for three days and ELISA measurements were done again to determine the decrease in the IgG levels in rats. Figure 5 shows the graphical presentation of the decrease in IgG levels in rats after treatment with the samples.

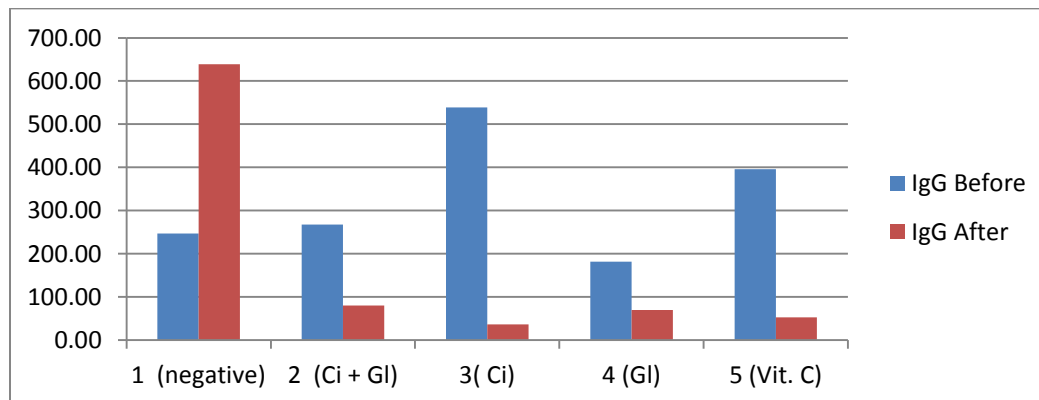


Figure 5: Immunoglobulin G levels of *R. albus* before and after treatment of the test drug materials

Analysis of variance result was $F(3,8) - 174.115$, $p < .001$. Table 2 shows the post-hoc analysis of the mean differences of serum IgG levels of rats in the different treatments

Table 2: Post-hoc Analysis on the Effectiveness of Treatments to IgG Levels Using Hochberg

	Treatment	Mean Difference	Significance
CI	CI:GL	297.45	<0.0001
	GL	252.11	<0.0001
	+ control	164.89	<0.0001
+ control	CI:GL	132.55	<0.0001
	GL	87.22	0.001
GL	CI:GL	45.33	<0.0001

Table 2 reveals that the differences of the treatments are statistically significant. This is shown by the computed p-values of $\leq .001$ for all the treatments. It can be inferred from the table that *C. indicum* was statistically more effective than all the other treatments. The data show that when taken separately, the two extracts were more effective, but when combined, there was a diminished effect. However, this does not mean to say that the combination was not effective, but that there was no synergism of the effect on the IgG levels in rats.

3.3.2 Immunoglobulin M

Before the rats were induced with infection, their baseline IgM levels were determined. Then, *E. coli* were injected and after 24 hours of infection, the IgM levels were determined by ELISA to check for the immune response of the rats as shown by the increase in their IgM levels. Then, introduction of the different treatments was done for three days and ELISA measurements were done again to determine the decrease in the IgM levels in rats. From the equation of the line of the calibration curve, the IgM values for each of the absorbance readings in ELISA for the treatments were computed. Figure 6 shows the graphical presentation of the decrease in IgM levels in rats.

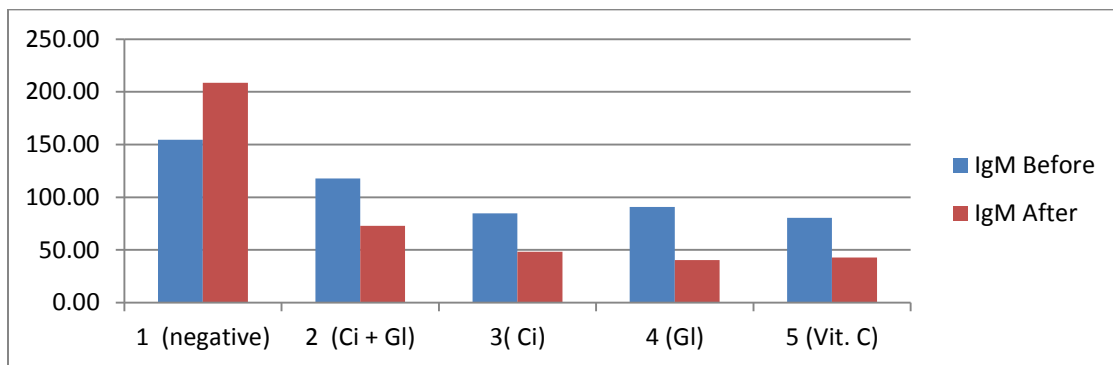


Figure 6: Immunoglobulin M levels of *R. albus* before and after treatment of the test drug materials

ANOVA shows no significant difference in the different treatment as shown by the computed values of $F(3,8) = 3.998$, $p = .148$. Table 3 shows the post-hoc analysis of the serum IgM levels in rats treated with the extracts.

Table 3: Post-hoc Analysis on the Effectiveness of Treatments to IgM Levels Using Games-Howell Test

Treatment	Treatment	Mean difference	Significance
CI:GL	CI	127.88	0.324
	GL	71.66	0.649
	+ control	127.03	0.329
GL	CI:GL	71.66	0.649
	CI	56.23	0.223
	+ control	55.37	0.139
+ control	CI	0.86	1.000

The table reveals that the differences in the effectiveness of the different treatment are statistically insignificant at $\alpha 0.05$.

3.3.3 Immunoglobulin A

In this study, there was already an increase in immunoglobulin levels in rats after 24 hours of *E. coli* injection as shown in the ELISA measurements. Then, introduction of the different treatments was done for three days and ELISA measurements were done again to determine the decrease in the IgA levels in rats. From the equation of the line of the calibration curve, the IgA values for each of the absorbance readings in ELISA for the treatments were computed. Figure 7 shows the graphical presentation of the decrease in IgA levels in rats.

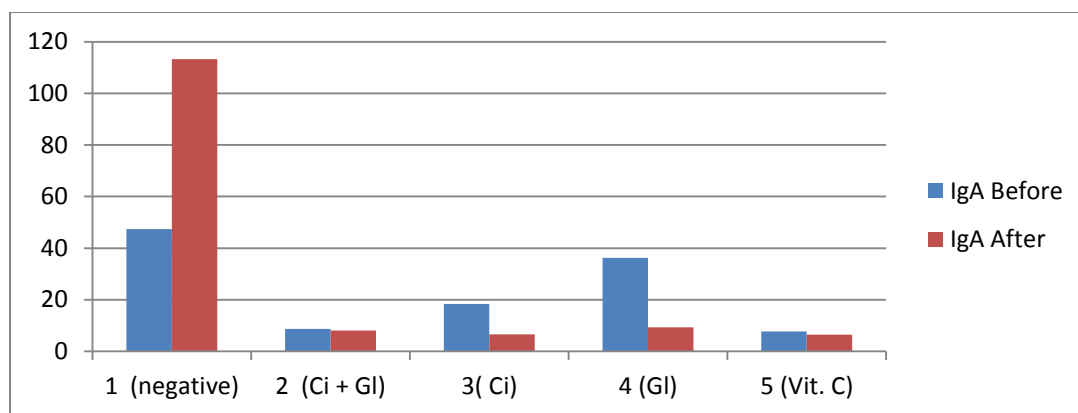


Figure 7: Immunoglobulin A levels of *R. albus* before and after treatment of the test drug materials

ANOVA results show significant differences in the treatment with $F(3,8) = 8.458$, $p = 0.007$. Table 4 shows the post-hoc analysis of the different treatments on rat serum IgA.

Table 4: Post-Hoc Analysis for the Different Treatments on IgA Using Hochberg Test

Treatment	Treatment	Mean Difference	Significance
CI	CI:GL	30.78	0.132
	+ control	30.05	0.144
GL	CI:GL	47.87	0.016
	CI	17.09	0.61
	+ control	47.13	0.018
+ control	CI:GL	0.74	1.000

It can be gleaned from the table that 2 among the treatments are statistically significant. *G. lucidum* is significantly more effective than that of the combination and the positive control, Vitamin C with computed p-values of 0.016 and 0.018 respectively.

The application of *G. lucidum* on immunoglobulins was done by Liu *et al.*, (2015) who showed only the role of the *G. lucidum* hot water extract on increasing the immunoglobulins in cows' milk. Their results showed no significant differences in milk IgG, IgA, or IgM, but serum IgA concentration was significantly higher in experimental groups compared with control group. In contrast, this study showed that the beta-glucan from *G. lucidum* acts as immune modulator by decreasing the IgG, IgM and IgA serum levels in infected rats.

The results of this study can be compared with some of the findings of other researchers. In the research of Cheng *et al.* (2005), they found that *C. indicum* flower has anti-inflammatory, and cellular immunomodulatory and mononuclear phagocytic activities on mice splenic cells by increasing the IgM and IgG levels of the splenic cells. In contrast, Park *et al.* (2012) found that topical application of *C. indicum* suppresses serum IgE and IgG levels in mice induced with atopic dermatitis. This means that the immunoglobulin levels can be modulated by *C. indicum* either by increasing in response to counteracting inflammation or lowering when infection is induced. In the present study, infection was done, so it was expected that IgG, IgM and IgA levels were decreased by *C. indicum*. So far, no studies were done to establish the IgA response on *C. indicum*, thus the major contribution of this study was established. Compared to the different studies mentioned, this study established the role of *C. indicum*, *G. lucidum* and their

combination in modulating the three types immunoglobulins (A, G and M) in infection-induced mice.

3.4 Lymphocyte Test Measurement

The lymphoproliferative effect of natural products like plant extracts had been evaluated by many researchers especially for addressing supplementation for immunocompromised individuals (Gomez-Flores et al., 2008) and also beta glucan from fungal extracts for patients with advanced breast cancer (Demir et al., 2007).

Figure 8 shows that there is an increase in the number of lymphocytes in *R. albus* after treatment of the different test drugs. Statistically, the increase was significant ($t=2.659$, sig. 0.019). It can be gleaned from the data that the highest increase was seen on Vitamin C followed by the ganoderma and chrysanthemum combination with 20% and 15% increase respectively. However, it was shown by ANCOVA that there was no significant differences in the effects of the treatments/drugs at $\alpha 0.05$ ($F=0.805$, sig. 0.530) suggesting that all treatments were comparable in their effects in proliferating lymphocytes in response to inflammation.

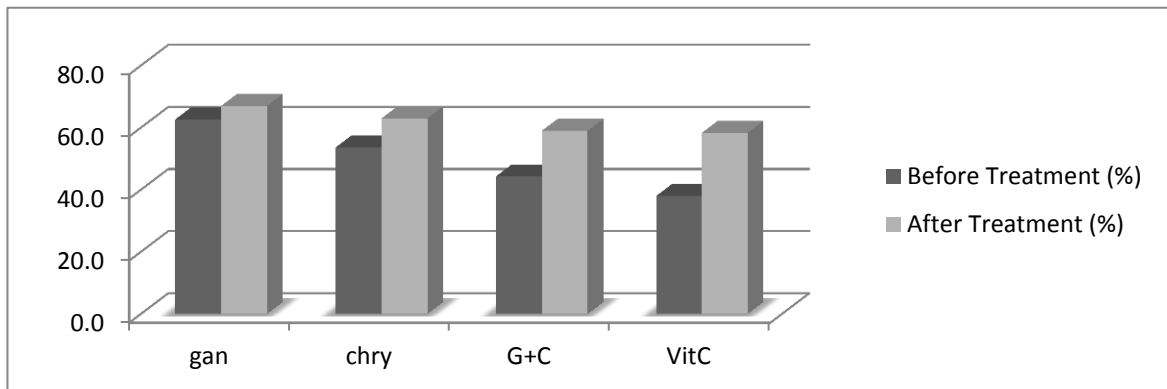


Figure 8: Percentage lymphocytes on *R.albus* before and after treatment of drug materials following inflammation

In this study, rats were induced with inflammation and were treated with ganoderma and chrysanthemum. Vitamin C was the positive control for immunomodulation. When taken alone, beta glucans from *G. lucidum* increased lymphocytes from 63-67%, *C. indicum* extract increased from 54-63%, the combination of *G.lucidum* and *C. indicum* increased from 44-59% while that of Vitamin C increased from 38-58%. According to the University of Pennsylvania School of Veterinary Medicine (2002), the normal values for rat lymphocytes were in the range of 50-70%.

It is noteworthy that in the set-up for the combination (GL +CI), the lymphocytes values became normal.

4. Conclusion

In the ongoing search for natural products to address lifestyle diseases like cardiovascular disease coupled with immunosuppression, the combination of *G. lucidum* beta glucans and *C. indicum* flower extract addresses both and offers a cheap, safe and effective alternative natural medicine. This pre-clinical screening shows promise for pharmaceutical development. Future undertakings include the assay and use of the drug combination in a clinical setting so that its effectiveness can be evaluated on humans. Both the medicinal and economic benefits from this fungi and the plant will be harnessed in the future.

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6. Conflict of Interest

The authors declare no conflict of interest.

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