Antioxidant and Anti-Inflammatory Activity and Cytotoxicity of Ethanol Extracts from *Rhynchosia nulubilis* Cultivated with *Ganoderma lucidum* Mycelium

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ABSTRACT: In this study, in order to verify the use of mycelium as a new functional material, *Rhynchosia nulubilis* was cultivated with *Ganoderma lucidum* mycelium, and the biological activity of the culture extract was evaluated. Measurements of the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging activity showed that the ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (RNGM) had significantly higher radical scavenging activity compared to the ethanol extract from *Rhynchosia nulubilis* (RN). The growth inhibition rate of RNGM against HeLa cells was 93.93% at 400 µg/mL in the 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium assay. RNGM effectively decreased nitric oxide production in lipopolysaccharide-treated RAW264.7 macrophage cells. The total polyphenol, total flavonoid, and the β-glucan contents of RNGM were higher than those of RN. These results demonstrated that increased total polyphenols, flavonoids, and β-glucan of *Rhynchosia nulubilis* during cultivation with the *Ganoderma lucidum* mycelium could increase the antioxidant and anti-inflammatory activity and cytotoxicity of cancer cells.

Keywords: *Rhynchosia nulubilis*, *Ganoderma lucidum* mycelium, antioxidant activity, anti-inflammatory, cytotoxicity

INTRODUCTION

Black beans contain more than 40% of protein with excellent nutritional properties (1). Black beans also have high biological activity, including antioxidant activity (2). In addition, it was reported that functional materials such as saponins, isoflavones, and anthocyanins in black beans aid in the prevention and improvement of cerebrovascular and cardiac disorders (2,3). Especially, it has been reported that black beans, which contain large amounts of genistein and daidzein in isoflavones, have high antioxidant activity causing elimination of oxygen radicals and inhibition of the production of carcinogenic substances, leading to anticancer activity against breast, rectal, and prostate cancers (1,4). *Rhynchosia nulubilis*, also known as Yakkong, Jwinunikong, and Tawonkong, is a species of black bean with a seed size of 5~7 mm and is a one-year black bean species harvested in October (5). *Rhynchosia nulubilis* contains a higher amount of isoflavones than other black beans, and it is known to be helpful in preventing and treating diseases such as high blood pressure, diabetes, anti-aging, osteoporosis, and senile dementia (6).

Mushrooms aid in enhancing the physical constitution, antiviral activity, cholesterol lowering effects, hypotensive effects, antithrombotic activities, immune functions, and antitumor activities (7,8). The fruit body as well as the mycelium of mushrooms is reported to have significant effects on biological activities, which has generated considerable industrial interest in the fields of exploration, development, and usage of bioactive substances. Among them, *Ganoderma lucidum* is a basidiomycete of the family Polyporaceae, and it is called the herb of eternal life (Bullocho). It is widely used in medications for disorders such as asthma, bronchitis, arthritis, and high blood pressure (7,8). In addition, it is reported to have various biological activities including antitumor, anticancer, and antioxidant activities as well as strengthening the immune system (9-11). However, the use of *Ganoderma lucidum* as a functional food has been limited because it is expensive. Therefore, the functions and usage of the mycelium, which are reported to be relatively cheaper and pharmacologically effective, are receiving increased attention. The mushroom mycelium grows on dead trees or organic substrates and absorbs the necessary nutrients through

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the secretion of cellular enzymes. It is expected that *Ganoderma lucidum* may contain novel active compounds that will aid in the enhancement of biological activities by exerting synergistic effects through fungal biotransformation. Interestingly, compared to the mycelium cultured in the existing general medium, some natural products have been shown to have more active compounds and better biological activities. Previous studies using mulberry (12) and medicinal herbs (13) as natural media for mycelium cultivation showed an increase in biological activities. *Rhynchosia nulubilis* can be used as a medium for cultivation because it contains valuable nutrients. The purpose of the present study was to investigate the changes in biological effects including antioxidant activities and cytotoxicity to cancer cells and to analyze the functional components after culturing *Rhynchosia nulubilis* with *Ganoderma lucidum* mycelium.

MATERIALS AND METHODS

Materials

Rhynchosia nulubilis was purchased from Yangnyeongsi in Daegu, Korea. *Ganoderma lucidum* mycelium KFRI 1594 was supplied by the Korean Forest Research Institute (Wanju, Korea) and incubated at 25°C in potato dextrose agar (PDA) followed by incubation at 4°C. This was used for subculturing every 4 weeks.

Chemicals

For cultivation, PDA and potato dextrose broth (PDB) were purchased from Acumedia (Lansing, MI, USA). For the antioxidant tests, 2,2-diphenyl-1-picrylhydrazyl (DPPH), L-ascorbic acid, hesperetin, quercetin, and Folin-Ciocalteu's phenol reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Also, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was purchased from Fluka (Heidelberg, Germany). For the cytotoxicity tests, 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium (MTT) and sulforhodamine B (SRB) were purchased from Sigma Chemical Co.. Roswell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's modified eagle medium (DMEM), and penicillin-K-streptomycin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA), and fetal bovine serum (FBS) was purchased from Gibco BRL (Paisley, UK).

Preparation of extracts

Ganoderma lucidum mycelium was inoculated in a PDA plate and incubated at 25°C for 7 days. After incubation, the mycelial disk was cut with a sterilized cork borer (8 mm) into 5~6 disks. The mycelium disks were then transferred to PDB and incubated with constant shaking at 25°C for 7 days. After incubation, the *Ganoderma luci-*

dum mycelium was homogenized in a stomacher (400 Mark II, Seward Laboratory Systems Inc., Port St. Lucie, FL, USA) and transferred to *Rhynchosia nulubilis*, which was immersed in distilled water for 1 h and sterilized. This was followed by incubation at 25°C for 10 days. *Rhynchosia nulubilis* without inoculation with *Ganoderma lucidum* mycelium was immersed in distilled water for 1 h and sterilized by autoclaving at 121°C for 15 min. *Rhynchosia nulubilis* and *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (100 g) were transferred to 80% ethanol (1 L) and continuously shaken for 24 h at 25°C. The ethanol extracts were filtered using a filter paper (Toyo No. 2, Advantec, Tokyo, Japan) and concentrated using a rotary evaporator (EYELA, Rikakikai Co., Tokyo, Japan). The extract of *Rhynchosia nulubilis* (RN) and the extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (RNGM) were then freeze-dried and stored at -18°C.

DPPH assay

The DPPH radical scavenging activity of the samples was determined according to the method by Zhang et al. (14). The sample was diluted with 0.1 M sodium acetate buffer (pH 5.5), and an aliquot of 1 mL was mixed with 2 mL of 7.5×10^{-5} M DPPH solution. After incubation for 30 min at 37°C, the absorbance was measured with a UV/visible spectrophotometer (Du 800, Beckman Coulter, La Habra, CA, USA) at 517 nm. The DPPH radical scavenging activity (%) was calculated using the equation below, and the measurements were repeated 3 times.

$$\text{DPPH radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

ABTS assay

The scavenging activity of the sample on the ABTS radical cation was estimated according to the method by Joung et al. (15). The radicals are generated by a reaction between 7 mM ABTS and 2.45 mM potassium persulfate aqueous (1:1) in the dark at 25°C for 12 h. The ABTS⁺ solution was diluted with 5 mM potassium phosphate buffer (pH 7.4) to the absorbance value of 0.7. The ABTS⁺ reagent (4 mL) was allowed to react with the sample extracts (40 µL) for 1 min. The absorbance was then measured at 413 nm using the spectrophotometer. Vitamin C was used in the comparison group, and the scavenging activity of the sample on the ABTS radical scavenging activity was calculated by the equation below, and the measurements were repeated 3 times.

$$\text{ABTS radical scavenging activity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted according to the method by Kim et al. (16). A working reagent was produced with 300 mM sodium acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-*s*-triazine in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution in a 10:1:1 ratio. The reaction mixtures containing the sample and FRAP reagent were mixed in a 7:1 (v/v) ratio. The colored product was then measured at 593 nm using a spectrophotometer. A standard curve was prepared using working solutions of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and the results were expressed as FeSO_4 eq. $\mu\text{M}/\text{mL}$ of sample.

Cell culture

The 4 types of cell lines used in this study were A549 (human lung adenocarcinoma epithelial cell line), Hep3B (human hepatocellular carcinoma cell line), HeLa (human uterine carcinoma cell line), and HeLa229 (human normal cell). All cell lines were obtained from the Korean Cell Line Bank (Seoul, Korea). The A549, HeLa, and HeLa229 cell lines were cultured in RPMI 1640 medium, and the A549 cell line was cultured in DMEM. Both media were supplemented with 10% FBS (v/v) and 1% antibiotics (penicillin-streptomycin) (v/v). The cell lines were incubated at 37°C in 95% humidity and 5% CO_2 .

MTT assay

The cytotoxicity of the extracts against the 4 cancer cell lines was measured by the MTT assay (17). The cell lines were grown in the medium and were seeded in 96-well plates at a density of 5×10^4 cells/mL. After incubation for 24 h at 37°C in a 5% CO_2 , 80 μL of medium was removed, and each sample was added to each well and incubated at 37°C in a 5% CO_2 incubator for 48 h. The MTT solution (20 μL) was added to each well and incubated for 4 h. After incubation, the supernatants were removed from each well to generate formazan crystals, which were melted to 150 μL of DMSO : ethanol (1:1) and cultured on a shaker for 30 min. The absorbance was measured at 550 nm using an ELISA reader (Versamx, USA). The inhibition of the cancer cell viability of the extracts against the 4 cancer cell lines is shown as cytotoxicity using the equation below, and the average value after triplicate readings was used in the following equation.

$$\text{Cytotoxicity (\%)} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

SRB assay

The SRB assay was performed according to the method by Zhang et al. (17). The incubated cancer lines were seeded onto 96-well plates at a density of 5×10^4 cells/mL in 100 μL and incubated at 37°C in a 5% CO_2 incubator for 24 h. After incubation, 100 μL of sample was added

to each well and incubated at 37°C in 5% CO_2 for 48 h. The supernatant was then discarded, and 100 μL of 10% trichloroacetic acid (TCA) was added to each well. After incubation at 4°C for 1 h, the TCA was removed and rinsed 5 times with distilled water. After drying at 25°C, 100 μL of SRB (0.4% w/v in 1% acetic acid) was added to each well and incubated for 30 min at 25°C for the staining process. Next, each well was washed 5 times with 1% (v/v) acetic acid, and 100 μL of 10 mM Tris buffer (pH 10.5) was added to each well. The absorbance was measured at 540 nm, and cytotoxicity was calculated with the equation used in the MTT assay. All data were reported as the mean of three independent readings.

Cell viability and nitric oxide (NO) determination

The MTT assay was used to assess whether the sample is cytotoxic to RAW264.7 cell lines (17). The nitrite concentration in the medium was measured by the Griess reagent and used as an indicator of NO synthesis in the cells (18). The RAW264.7 cell line was cultured, and 100 μL of 1×10^5 cells/mL solution was added to each of the 96-well plates to measure NO and incubated for 24 h. Then, 20 μL of sample with different concentrations and 1 $\mu\text{g}/\text{mL}$ lipopolysaccharide (LPS) were added to each well and incubated for 24 h under 5% CO_2 at 37°C. After incubation, 100 μL of culture supernatant was mixed with the Griess reagent [1% sulfanilamide in 5% phosphoric acid : 0.1% naphthylethylenediamine dihydrochloride in water = 1:1 (v/v)] and incubated for 10 min at room temperature. Absorbance was measured at 570 nm, and the nitrite concentration was determined using a standard curve of sodium nitrite prepared in the culture medium.

Total polyphenol and total flavonoid contents

The total polyphenol content was determined using the Folin-Denis method (14) with slight modifications. An aliquot of 0.5 mL of the sample was oxidized with 0.3 mL of Folin-Ciocalteu's phenol and neutralized by the addition of 10 mL of 7% sodium carbonate. Following incubation for 2 h, the absorbance of the reaction mixture was measured at 750 nm using the spectrophotometer against a blank of distilled water. Gallic acid was used as the standard, and the total polyphenol contents were expressed as mg gallic acid equivalents (mg GAE/g).

The total flavonoid content of the samples was determined using aluminum chloride, according to the method by Moreno et al. (19). A 0.5 mL aliquot of the sample was mixed with 1 mL of 10% aluminum nitrate, 1 mL of 1 M potassium acetate, and 4.3 mL of 80% ethanol. Following incubation for 40 min, the absorbance of the reaction mixture was measured at 510 nm using a UV/visible spectrophotometer. Quercetin was used as the standard, and the flavonoid contents were expressed as mg

quercetin acid equivalents (mg QE/g).

β -Glucan content

The β -glucan contents of the extracts were measured using the β -glucan enzymatic assay kit (Megazyme International Ireland Ltd., Bray, Ireland).

Statistical analysis

Statistical analysis was performed with SPSS ver. 10 package program (SPSS Inc., Chicago, IL, USA). The results are expressed as mean $\pm$ standard deviation (SD), and a 2-tailed value of $P < 0.05$ was considered statistically significant. A difference in the variables among different concentrations was analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Differences in the total polyphenol, total flavonoid, and β -glucan contents between two samples were analyzed using the t -test.

RESULTS AND DISCUSSION

DPPH assay

The radical scavenging activity of RN and RNGM is shown in Fig. 1. RN showed radical scavenging activity of 3.50, 10.20, 16.41, 29.51, 47.15, 59.95, and 61.33% at 10, 100, 200, 400, 600, 800, and 1,000 $\mu\text{g/mL}$. RNGM showed radical scavenging activity of 3.19, 18.87, 21.68, 33.31, 55.49, 66.71, and 70.54% at the same concentrations. RNGM had a DPPH radical scavenging ability at 100–1,000 $\mu\text{g/mL}$, which was significantly higher than that of RN. These results were similar to those reported

Fig. 1. The electron donating ability of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Values with different letters are significantly different among the different concentrations in the same sample (A–E) and among the different samples in the same concentration (a–c) by Duncan's multiple range test ($P < 0.05$). A.A., ascorbic acid; RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

by Kim et al. (20), who showed that DPPH radical scavenging ability of mushroom mycelium culture that was cultivated with a citrus-added medium increased after cultivation. It is believed that the antioxidant ability of RNGM increased due to an increase of antioxidant substances during cultivation.

ABTS assay

The ABTS radical scavenging activities of RN and RNGM are shown in Fig. 2. The RN showed 13.81, 50.98, 64.74, 78.45, and 85.90% activities at different concentrations (10, 100, 200, 400, and 500 $\mu\text{g/mL}$), while RNGM showed activities of 17.62, 53.84, 75.73, 92.05, and 95.82%. The ABTS radical scavenging activities of RNGM were higher than those of RN at the concentration of more than 200 $\mu\text{g/mL}$, which was identical to the DPPH assay result. Joung et al. (17) reported that when *Ganoderma lucidum* mycelium was cultured with ginseng, the ABTS radical scavenging activity was significantly increased compared to that of the original material, ginseng extract. Bae and Moon (3) reported a higher increase in the antioxidant activities of beans fermented by microorganisms compared to unfermented beans. These findings are consistent with those of the present study, which shows that the antioxidant activity had increased by the process of biotransformation when the mycelium was cultivated. According to Kim et al. (21), various enzymatic reactions in mushroom mycelium resulted in biotransformation from low-activity to high-activity antioxidants. Therefore, it is considered that the higher antioxidant activity of RNGM compared to that of RN, as observed in the present study, is attributed to an increase in the amount of strong anti-

Fig. 2. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS) radical scavenging activity of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Values with different letters are significantly different among the different concentrations in the same sample (A–E) and among the different samples in the same concentration (a–c) by Duncan's multiple range test ($P < 0.05$). A.A., ascorbic acid; RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

oxidant substances due to the microbial enzyme produced by the mycelium.

FRAP assay

The reducing capability of RN and RNGM in the FRAP assay is shown in Fig. 3. The FRAP values of RN and RNGM ranged from 5.39~83.57 FeSO₄ eq.μM and 41.76~104.18 FeSO₄ eq.μM, respectively. Especially, RNGM noticeably had a higher FRAP value than RN. These results are similar to those of the DPPH assay and ABTS assay. Consequently, the reducing power of extracts increased during the process of mycelium cultivation. This finding is consistent with the findings of Shon (22), who reported that the reducing power of the magnolia extract fermented with mushroom mycelium increased during the fermentation process. In addition, the study using *Acanthopanax senticosus* fermented with mushroom mycelium reported that extract of *Acanthopanax senticosus* fermented with *Ganoderma lucidum* mycelium had superior reducing power compared to the extract fermented with other mushroom mycelium (18). This shows that the reducing power of *Ganoderma lucidum* mycelium culture could increase during the fermentation process.

MTT assay

To compare the cytotoxicity of RN and RNGM on cancer cells, the MTT assay was used in A549, Hep3B, HeLa, and HeLa 229, and the results are shown in Fig. 4. The RNGM had higher cytotoxicity against Hep3B compared to RN at each concentration. The cytotoxicity rate of RNGM against HeLa at each concentration (50, 100, 200, 300, and 400 μg/mL) was 38.80, 48.22, 80.21, 88.30, and 93.93%, respectively. These results showed the highest

Fig. 3. Ferric ion reducing antioxidant power of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Values with different letters are significantly different among the different concentrations of RN (A-D) and among the different concentrations of RNGM (a-d) by Duncan's multiple range test ($P < 0.05$). The asterisks indicate a significant difference between RN and RNGM at each concentration by t -test (* $P < 0.05$ and ** $P < 0.01$). RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

cytotoxicity against all the cancer cells tested. The growth inhibition rate of RN against A549 was 73.45% at the highest concentration i.e., 400 μg/mL, whereas the growth inhibition rate of RNGM was 89.59%. Likewise, against every cancer cell, RNGM had significantly higher cytotoxicity than RN, indicating that anticancer activity increased through the cultivation of mycelium. This is in agreement with the study by Park et al. (11) on the cytotoxicity of *Lentinus edode* mycelium extracts, which suggested that the mycelium extract concentration-dependently inhibits liver proliferation. In addition, Bae and Ye (23) found that *Halls honeysuckle* extracts cultured with *Lentinus edode* mycelium had cytotoxicities of 23.60~88.44%, compared to that of *Halls honeysuckle* extracts without cultivation i.e., 23.90~26.73% against Hep3B, thereby indicating that *Lentinus edode* mycelium influenced the inhibition of cancer cell proliferation. Thus, cultivation with mycelium may have a significant effect on anticancer activity and should be further investigated for the possibility of anticancer effects in *in vivo* experiments. Meanwhile, the growth inhibition test against normal human cervical cells HeLa 229 was performed to investigate the cytotoxicity of the sample itself on the normal cells. It was found that the sample had less than 25% cytotoxicity against HeLa 229 at the highest concentration.

SRB assay

The cancer cell growth inhibition effect of RN and RNGM by the SRB assay is shown in Fig. 5. The SRB assay results showed that all extracts concentration-dependently inhibited the cell growth of Hep3B and HeLa, similar to the MTT assay. At each concentration (50, 100, 200, 300, and 400 μg/mL), RN showed inhibition rates of 4.93, 10.58, 39.80, 64.95, and 73.23% against A549 while RNGM showed inhibition rates of 5.14, 14.76, 57.89, 75.34, and 84.86%. At the same concentration, RNGM had a higher inhibition rate of 12.10~86.42% against Hep3B than RN with 1.37~72.03%, thereby indicating that the anticancer effects against Hep3B increased after cultivation with *Lentinus edode* mycelium. Although the inhibition rate against A549 was lower than that against HeLa and Hep3B, RNGM showed the cytotoxicity of 84.86% on A549 at 400 μg/mL. Consequently, anticancer activities of each extract for 3 types of cancer cells increased in a concentration-dependent manner, and the cytotoxicity increased after cultivation with *Lentinus edode* mycelium. Kim et al. (24) reported that the extract from a culture of *Coriolus versicolor* Quel and *Ganoderma lucidum* mycelium had high anticancer activity. Zhang et al. (14) also found that the ethanol extract of waxy sorghum cultured with *Phellinus linteus* mycelium had higher cytotoxicity, compared to that of non-cultured waxy sorghum. In the present study, it is thought that the increased cytotoxicity of RNGM is due to the influence of cultivation

Fig. 4. Cytotoxicity of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* on the growth of various using the MTT assay. Values with different letters are significantly different among the different concentrations of RN (A-E) and among the different concentrations of RNGM (a-e) by Duncan's multiple range test ($P < 0.05$). The asterisks indicate a significant difference between RN and RNGM at each concentration by t -test (* $P < 0.05$ and ** $P < 0.01$). RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

with mycelium. The cytotoxicity of each extract on normal cells, HeLa 229, was not very high ($< 30\%$) at every concentration.

Cytotoxicity evaluation on macrophages

To set the experimental conditions for evaluating the anti-inflammatory efficacy of each extract, the cytotoxicity was checked by treating RN and RNGM against RAW264.7 macrophages at different concentrations (50, 100, 200, 300, 400 and 500 µg/mL) (Fig. 6A). As a result, both samples, RN and RNGM, had cell viabilities of more than 90% at the concentration of 400 µg/mL and below, which indicates that they had no cytotoxicity against RAW264.7 macrophages at these concentrations. However, the cytotoxicity against RAW264.7 was identified at 500 µg/mL. Based on this result, experimental conditions were set to 5 concentrations (50, 100, 200, 300, and 400 µg/mL) in order to analyze the anti-inflammatory efficacy of each extract.

NO production of macrophage cell lines

The present study investigated the influence of each extract on NO production by treating the extracts on macrophages in which inflammation was caused by bacterial

LPS (Fig. 6B). The production of NO of LPS-treated macrophage cells increased 3 times compared with that of normal cells. The increased production of nitrite by LPS did not decrease in the groups treated with RN and RNGM at the concentration of 50 µg/mL. However, a significant decrease of nitrite was observed in the treatment groups at concentrations of more than 100 µg/mL. The group treated with RN extracts at the concentrations of 100, 200, 300, and 400 µg/mL had NO production of 20.08, 16.57, 12.66, and 10.05 µM; The RNGM group had NO production of 19.98, 12.95, 11.05 and 7.85 µM. Likewise, at every concentration except 50 µg/mL, the NO production significantly decreased in the RNGM group compared to the RN group. RNGM decreased 39% of the produced NO at the concentration of 400 µg/mL compared to the LPS-treated macrophage cells. Jung et al. (18) reported that the extracts from germinated brown rice cultured in *Phellinus linteus* mycelium culture fluid showed higher inhibitory effects on NO production than that of brown rice or germinated brown rice. This is in agreement with the findings of the present study, suggesting that inhibitory activity of NO production may be increased by active ingredients produced in the process of cultivation of mycelium. Macrophages are important

Fig. 5. Cytotoxicity of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium on the growth of various using the SRB assay. Values with different letters are significantly different among the different concentrations of RN (A-E) and among the different concentrations of RNGM (a-e) by Duncan's multiple range test ($P < 0.05$). The asterisks indicate a significant difference between RN and RNGM at each concentration by t -test (* $P < 0.05$ and ** $P < 0.01$). RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

for natural immune responses since they explore, decompose, and eliminate foreign substances from outside of the body, and they also recognize antigens from outside

and make antibodies against antigens (18). *L*-arginine is changed to NO and citrulline by NO synthase, which consists of constitutive NO synthase (cNOS) and inducible

Fig. 6. Cytotoxicity of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium in RAW264.7 cells (A) and nitric oxide production in LPS-induced RAW264.7 cells (B). Values with different letters are significantly different among the different concentrations of RN (A,B) and among the different concentrations of RNGM (a,b) by Duncan's multiple range test ($P < 0.05$). The asterisks indicate a significant difference between LPS and extract (RN or RNGM) by t -test (* $P < 0.05$ and ** $P < 0.01$). The hash marks indicate a significant difference between RN and RNGM at each concentration by t -test (# $P < 0.05$). RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium.

NO synthase (iNOS). The cNOS is known to develop in cells like vascular endothelial cells at a constant level and consistently creates low levels of NO, which are necessary for physiological functions. On the other hand, iNOS is believed to develop in epithelial cells and leukocytes by LPS or cytokines and produces excess NO (25). Therefore, the excess production of NO during inflammation reactions may result from iNOS and too much NO produced *in vivo* may deteriorate inflammation, leading to tissue damage, genetic variance, and neural damage (17). Therefore, the amount of NO secreted from macrophages can indirectly indicate the production amount of molecules deteriorating inflammation. However, further studies on the mechanisms of the expression of iNOS as an important inflammation mediator are needed to determine the effects of RNGM on the production of reactive nitrogen metabolites.

Total polyphenol and flavonoid contents

The total quantity of polyphenols in RN and RNGM is shown in Table 1. The total polyphenol contents are 5.76 mg GAE/g for RN and 7.87 mg GAE/g for RNGM, and RNGM had significantly more total polyphenols than RN. Kim et al. (16) studied the antioxidant activities of *Acanthopanax senticosus* fermented with mycelium and reported that the total polyphenol contents in ethanol extracts of fermented *Acanthopanax senticosus* with *Ganoderma lucidum* mycelium was 40.99 mg GAE/g, which was higher than ethanol extracts of *Acanthopanax senticosus*. In this regard, cultivation with *Ganoderma lucidum* mycelium is believed to be an excellent biotransformation process for producing polyphenol compounds. The contents of total polyphenol compounds including flavonoids, anthocyanin, and polyphenol compounds are important factors for antioxidant activities to remove free radicals.

Meanwhile, the total flavonoid content of RNGM was 4.32 mg QE/g, as shown in Table 1, with significantly higher contents than that of RN. It has been reported that flavonoids exert antioxidant and anticancer effects by the effective elimination of active oxygen (26). This result is in agreement to the findings of Kim et al. (27)

who reported that fermented ginseng with *Phellinus linteus*, *Ganoderma lucidum*, and *Hericium erinaceum* had higher contents of flavonoids than the raw material, ginseng. Therefore, it is considered that decomposition of phenolic compounds to low molecular weight compounds or the formation of new phenolic compounds such as melanoidin occur during the mycelium cultivation (28), which can increase the antioxidant activity of the culture. In this study, the increased total content of total polyphenols and flavonoids during *Ganoderma lucidum* mycelium cultivation is believed to influence the increase of antioxidant activity, which is demonstrated in the DPPH and ABTS assays.

β -Glucan content

The amount of β -glucans contained in RN and RNGM is shown in Table 1. The β -glucan content was 4.34% for RN and 13.26% for RNGM, and RNGM had significantly more β -glucans than RN. Lee et al. (29) showed that the β -glucan content was approximately 10.64% in the mycelium of *Sparassis crispa* Wulf, and this was lower than RNGM. The β -glucans in mushrooms are recognized as functional materials that offer considerable beneficial impacts in human health, such as antioxidant, and show antitumor activity and immunomodulatory effects (30). Further, the biological activities of mushroom β -glucans have been reported in previous studies (31). In addition, Huang et al. (32) reported that β -glucan isolated from *Poria cocos* mycelia had cytotoxicity against Sarcoma 180 cell.

These results suggest that *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium can enhance physiological activities including antioxidant activity, cytotoxicity to cancer cells, and anti-inflammatory activity compared to non-cultivated *Rhynchosia nulubilis*.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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Table 1. Contents of total polyphenols, total flavonoids, and β -glucan of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium

Sample	Total polyphenols (mg GAE/g)	Total flavonoids (mg QE/g)	β -glucan (%)
RN	5.76 $\pm$ 0.53	3.89 $\pm$ 0.13	4.34 $\pm$ 0.13
RNGM	7.87 $\pm$ 0.81*	4.32 $\pm$ 0.53	13.26 $\pm$ 0.39*

The asterisks indicate a significant difference between RN and RNGM by *t*-test (**P*<0.05).

RN, ethanol extract from *Rhynchosia nulubilis*; RNGM, ethanol extract from *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium; GAE, gallic acid equivalents; QE, quercetin acid equivalents.

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