Analysis of Antioxidant and Anti-Inflammatory Activities of Solvent Fractions from *Rhynchosia nulubilis* Cultivated with *Ganoderma lucidum* Mycelium

Mihye Park and Meera Kim

Department of Food Science and Nutrition, Kyungpook National University, Daegu 41566, Korea

ABSTRACT: In this study, the crude ethanol *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (RNGM) extract was solvent fractionated with organic solvents such as *n*-hexane, chloroform, ethyl acetate, and water. The anticancer activities, anti-inflammatory activity total polyphenols, total flavonoids, isoflavones, and β -glucan of the solvent fractions of RNGM were studied. The ethyl acetate fraction showed the highest 2,2-diphenyl-1-picrylhydrazyl scavenging activity of 76.60% at 800 μ g/mL. The ethyl acetate fraction also showed higher antioxidant activity in 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and ferric reducing ability of plasma assays compared to the other fractions. In addition, this study confirmed that the ethyl acetate fraction strongly inhibited nitric oxide production. The ethyl acetate fraction had the highest amount of total polyphenol and total flavonoid (65.33 mg gallic acid equivalent/g and 18.50 mg quercetin equivalent/g, respectively). The ethyl acetate fraction (13.02%) showed the highest amount of total β -glucan, followed by the water (6.32%), chloroform (1.43%), and *n*-hexane fraction (0.85%). Therefore, it is suggested that the ethyl acetate fraction of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium may be potential natural sources for nutritional and pharmaceutical applications.

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

INTRODUCTION

The worldwide increase in life expectancy and the associated rise in the aged population have greatly widened interest in healthy aging. Thus, research on natural antioxidant and anti-inflammatory substances that prevent aging and inflammatory diseases is being actively conducted. Artificial and synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene have been used for their antioxidant effects and preservation of foods. They, however, also increase the risk of cancer by their accumulation in the body (1). Furthermore, recent studies of conventional anti-inflammatory drugs such as cortisol-synthesizing agents have shown numerous side effects such as ulcerative colitis (2). In addition, natural anti-inflammatory substances that inhibit the generation and secretion of inflammation mediators such as nitric oxide (NO) and tumor necrosis factor- α have been actively studied (2).

The activity and yields of active substances are top priorities for the extraction process of natural products as

well as for studying their biological activity. Physiologically active plant ingredients have a variety of molecular structures and different polarities and, therefore, various solvents such as methanol, hexane, and water have been used for their extraction. Solvent fractionation using solvents with different polarities is the simplest and the most widely used method for the identification of substances (3,4).

The biological activities of medicinal mushrooms have been reported, including the antioxidant effects and improvement of the immunity system (3). *Ganoderma lucidum* is a widely used medicinal mushroom in traditional Asian medicine, which has bioactive compounds. Previous studies showed that *Ganoderma lucidum* has relatively high antioxidant, antitumor, immunomodulatory, and antihyperlipidemic blood glucose-lowering effects (5). However, the fruiting bodies of medicinal mushrooms, which are expensive and have long growth period, are the plant parts that are normally used. Therefore, numerous studies have been conducted on mycelia, which also have many advantages for use as food additives because of

Received 31 July 2017; Accepted 11 October 2017; Published online 31 December 2017

Correspondence to Meera Kim, Tel: +82-53-950-6233, E-mail: meera.k@knu.ac.kr

Copyright © 2017 by The Korean Society of Food Science and Nutrition. All rights Reserved.

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

their short growth period and ease of production using solid and liquid cultures. Mushroom mycelia are a group of higher fungi that grow on dead trees or organic substrates and absorb the necessary nutrients by the secretion of cellular enzymes; different kinds of edible mushroom mycelia exist in nature. Furthermore, they play an important role in the decomposition of organic matter in the natural ecosystem during their growth process. A recent study indicated that mycelia also have anticancer, immunostimulatory, antioxidant, and hypocholesterolemic effects (5,6).

Rhynchosia nulubilis is a variety of black bean that is widely used for the prevention of neuralgia, nephritis, and senile dementia. *Rhynchosia nulubilis* contains many biologically active compounds such as lecithin, trypsin inhibitors, phytate, phytosterols, saponins, and isoflavones (7). In particular, isoflavones are the major compounds found in *Rhynchosia nulubilis* and have four types of chemical structures including a glucose residue, which combines with β -1,4-glycoside to form a glycoside. Furthermore, fermented foods contain several glucose residues in the form of aglycones, which decompose glucose. *Rhynchosia nulubilis* contains a higher isoflavone content than other black beans and is known to be useful for preventing and treating diseases such as hypertension, diabetes, aging-related disorders, osteoporosis, and senile dementia (8,9).

We previously reported that the ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium showed higher antioxidant and anti-inflammatory activity than the uncultivated extract. In this study, the ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium was fractionated with different organic solvents to separate the active constituents, the antioxidant and anti-inflammatory activity of each fraction was examined, and the total polyphenol content was measured to determine its contribution to the potential natural antioxidant and anti-inflammatory properties of the extract.

MATERIALS AND METHODS

Fraction yields

The crude ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (RNGM) was prepared as follows: first the plant material was extracted with 10 times volume (v/w) of 80% ethanol, and then the crude extract was dissolved in water, followed by fractionation using a series of organic solvents, which were hexane, chloroform, and ethyl acetate, sequentially applied based on their polarity. Then, the RNGM extract, distilled water and hexane were transferred to a separatory funnel and shaken to ensure the mixture was well mixed. After

shaking, the separatory funnel was fixed on a stand without further shaking to allow the partitioning of the organic solvent and water fractions to occur. The hexane solvent fraction was removed, and similar volumes of the other organic solvents were sequentially added to the water fraction to obtain the chloroform and ethyl acetate fractions as well. The fractions were evaporated under reduced pressure, freeze-dried, and stored at -10°C , and the yields of the four extract fractions were expressed as percentages of the dry weight of the crude RNGM extract.

Antioxidant activities of solvent fractions

The antioxidant activities of the solvent fractions were estimated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) (10), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (11), and ferric reducing ability of plasma (FRAP) assays (12).

Anti-inflammatory activities of solvent fractions

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (10) was used to evaluate the cytotoxicity of the fraction samples against cultured RAW 264.7 cells. The nitrite in the culture medium was measured using the Griess assay and used as an indicator of NO synthesis in fraction-treated RAW 264.7 cells (13).

Determination of total polyphenol and flavonoid contents of solvent fractions

The total phenolic content was determined using the Folin-Denis method (14) with slight modifications. The total flavonoid content of the fractions was determined using the aluminum chloride test according to the method of Saiqa et al. (15).

Determination of β -glucan content

The β -glucan assay was conducted according to the β -D-glucan enzymatic assay kit (Megazyme International Ireland Ltd., Bray, Wicklow, Ireland).

Determination of isoflavones

The extraction of isoflavones from the solvent fractions was performed using the procedure described by Wang et al. (16) with slight modifications, and the stability of target isoflavones and complete conversion after hydrolysis were assessed. The isoflavones were determined as acid hydrolysates. In brief, 1 g of the solvent fraction was dissolved in 10 mL 80% methanol, sonicated for 2 h at 25°C , centrifuged at 8,000 rpm for 15 min, diluted to obtain a final concentration of 100 $\mu\text{g/mL}$, filtered using a 0.45 μm filter, and then analyzed using high-performance liquid chromatography (HPLC). For the hydrolysis procedure, 500 mg of the solvent fraction was added to 5 mL 1 N HCl, and the mixture was heated at 100°C for 1 h. Then, the samples were neutralized with 40% NaOH,

Table 1. Conditions for liquid chromatographic analysis of isoflavones

Description	Condition
Column	C ₁₈ , 5 μ m, 4.6×150 mm
Column oven temp.	35°C
Detector	Diode array detector, 254 nm
Mobile phase	A: 0.1% acetic acid in water B: 0.1% acetic acid in acetonitrile
Flow rate	1.0 mL/min
Injection volume	10 μ L
Composition of mobile phase	A : B=85:15 (0 min), 60:40 (30 min), 85:15 (40 min)

diluted to obtain a final concentration of 100 μ g/mL, and filtered using 0.45 μ m filters. Standard stock solutions at a concentration of 1 mg/mL were prepared by dissolving pure daidzein and genistein in 100% methanol. The concentrations of isoflavones were determined under the HPLC conditions shown in Table 1, and each experiment was repeated at least thrice.

Statistical analysis

Statistical analysis was performed with SPSS package program (version 10, Statistical Package for the Social Science, 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 continuous variables according to different concentrations and samples with antioxidant activity were tested using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

RESULTS AND DISCUSSION

Yield of solvent fractions

Natural products contain numerous bioactive compounds, which possess different polarities. Therefore, the solvent used for extraction has to complement the polarity of the target components. Additionally, some factors, should be considered when selecting the extraction solvents such as safety, cost effectiveness, and convenient recycling. Therefore, four solvents including *n*-hexane, chloroform, ethyl acetate, and water were chosen to investigate extraction yields. The yield of the four solvents were quite different, and the polarity-based fractionation revealed the following order: water (10.87%) > ethyl acetate (3.24%) > chloroform (2.61%) > *n*-hexane (0.86%). The yields of the polar solvents were higher than those of the non-polar solvents, and the lowest yield was from the *n*-hexane fraction.

Antioxidant activities of solvent fractions

DPPH assay: The DPPH radical scavenging activities of

Fig. 1. 2,2-Diphenyl-1-picrylhydrazyl radical scavenging ability of solvent fractions of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Different letters among the different concentrations of same sample (A-E) and the different samples of same concentration (a-e) are significantly different at $P < 0.05$ by Duncan's multiple range test.

the solvent fractions, which are shown in Fig. 1 revealed a dose-dependent effect. The ethyl acetate fraction showed the highest DPPH scavenging activity of 76.60% at a concentration of 800 μ g/mL. The chloroform and water fractions showed moderate scavenging properties of 56.92 and 34.71%, respectively, at the same concentration. In the DPPH test, the ethyl acetate fraction appeared to be a potential candidate for controlling free radical formation. This is similar with Ham et al. (17) who showed the ethyl acetate fraction of *Inonotus obliquus* crude ethanol extract exhibited the highest radical scavenging activity. The diethyl ether, ethyl acetate, and butanol fractions of the *Phellinus igniarius* mycelium extracts also showed DPPH scavenging activity of 82.6, 71.2, and 69.4 %, respectively. Lee et al. (18) reported the radical scavenging activity of diethyl ether and butanol fractions of *Ganoderma lucidum*, and that of the butanol fraction was 95%. Therefore, the antioxidant effect seems to be polarity dependent based on the related mushroom fractions that showed radical scavenging activity. The above result showed that the radical scavenging activities differed depending on the solvent fractions including the fruiting bodies and mycelium.

ABTS assay: In this study, the ABTS scavenging activity of the extract fractions increased concentration dependent manner (Fig. 2). Furthermore, the ethyl acetate fraction (86.61%) resulted in the highest ABTS radical scavenging activity, followed by chloroform, water, and hexane (81.84, 69.90, and 14.99%, respectively) at 1,000 μ g/mL. Compared to the antioxidant activities measured using the DPPH assay, the ABTS assay showed higher activities, which may be attributable to the difference in associated scavenging principles; the free radicals were scavenged in the DPPH assay while the positive ion radi-

Fig. 2. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of solvent fractions of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium. Different letters among the different concentrations of same sample (A-E) and the different samples of same concentration (a-e) are significantly different at $P < 0.05$ by Duncan's multiple range test.

icals were scavenged in the ABTS assay. The difference in scavenging, also affects the binding degree of each substrate, causing the differences in their ability to scavenge free radicals. The formation of excessive free radicals can cause the damage to cells, leading to dysfunctions such as atherosclerosis, myocardial infarction, cancer, and neurodegenerative disorders. However, natural antioxidant compounds are useful to improve various chronic diseases and for repairing the free radical damage to cells (11). In the ABTS test, it is demonstrated that the ethyl acetate fraction has good potential for controlling free radical formation.

FRAP assay: The FRAP assay is a simple, reproducible, rapid, and inexpensive method that measures the total antioxidant levels in a sample by determining their oxidation-reduction potential. The FRAP assay is based on the donation of an electron to convert the ferric ion (Fe^{3+}) to its ferrous (Fe^{2+}) form without the involvement of free radicals (12).

In the present study, FRAP values of the tested fractions varied at the range of 25~100 $\mu\text{g/mL}$ (Table 2). Within this range, the ethyl acetate fraction displayed

the highest FRAP values (22.89~102.26 FeSO_4 eq μM) followed by chloroform, water, and hexane fractions (14.53~83.27, 7.39~19.22, and 3.73~10.45 FeSO_4 eq μM , respectively). These results are in agreement with previous studies, which showed that polar fractions of plant extracts have higher antioxidant effects than the fractions with low polarity (19). Furthermore, we also demonstrated that the ethyl acetate fraction of RNGM showed a strong radical scavenging activity, probably due to its ability to chelate Fe^{2+} ions.

Anti-inflammatory activities of solvent fractions

The RAW 264.7 cells were incubated in the presence of the solvent fractions at wide concentration range (50, 100, 200, 300, and 400 $\mu\text{g/mL}$), and then the cell viability was evaluated using the MTT assay. We selected the entire concentration range for the subsequent experiment related to the inhibition of NO production because the cell viability was over 90% (Fig. 3A).

Inhibition of NO production by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells

NO is formed as a natural byproduct of the normal metabolism of oxygen. However, stimulation of cells by environmental stress such as ultraviolet radiation or heat exposure can lead to an inordinate increase in the accumulation of nitrite. This may cause the damage to cellular structures and further induce the inflammation by the oxidative stress (18).

In the present study, the cells were simultaneously treated with 1 g/mL LPS and different concentrations of the fractions. The RAW 264.7 cells were stimulated by LPS, and the nitrite that subsequently accumulated in the culture medium was determined. The results showed that the pre-treated cells induced with LPS released a higher level of NO in the medium, compared to the control. The results indicate that the inhibition of LPS-induced NO was concentration-dependent within most of the assayed fractions. The LPS-treated cells with ethyl acetate and water fractions generally decreased NO production as the each fraction concentration increased (Fig. 3B). This study confirmed that the level of concentration-dependent NO secretion was significantly different

Table 2. Ferric reducing ability of plasma of solvent fractions of ethanol extracts of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium (unit: FeSO_4 eq μM)

Fractions	Treatment concentration ($\mu\text{g/mL}$)			
	25	50	75	100
<i>n</i> -Hexane	3.73 $\pm$ 0.76 ^{Cd}	6.02 $\pm$ 0.80 ^{Bc}	8.93 $\pm$ 0.59 ^{Ad}	10.45 $\pm$ 0.86 ^{Ad}
Chloroform	14.53 $\pm$ 0.57 ^{Db}	38.92 $\pm$ 0.40 ^{Cb}	50.30 $\pm$ 0.51 ^{Bb}	83.27 $\pm$ 0.76 ^{Ab}
Ethyl acetate	22.89 $\pm$ 0.45 ^{Da}	56.50 $\pm$ 0.85 ^{Ca}	78.22 $\pm$ 0.13 ^{Ba}	102.26 $\pm$ 0.27 ^{Aa}
Water	7.39 $\pm$ 0.31 ^{Cc}	8.76 $\pm$ 0.65 ^{Cc}	14.22 $\pm$ 0.42 ^{Bc}	19.22 $\pm$ 0.64 ^{Ac}

Values are mean $\pm$ standard deviation (n=3).

Means with different letters in the same column (a-d) and the same row (A-D) are significantly different at $P < 0.05$.

Fig. 3. Effect of solvent fractions of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium on cell viability of RAW 264.7 cells (A) and nitric oxide production in lipopolysaccharide (LPS)-induced RAW 264.7 cells (B). Data represent the mean $\pm$ SD of three independent experiments. Different letters (A–D) among the different concentrations of same sample are significantly different at $P<0.05$ by Duncan's multiple range test. Asterisk indicates a significant difference from the value for the LPS-treated group by Student's t -test (* $P<0.05$ and ** $P<0.01$).

among the fractions. The ethyl acetate extract showed higher inhibition of NO production than the other fractions including the chloroform and hexane fractions. These results are consistent with the reports by Moro et al. (2), which described the anti-inflammatory activity of the ethanolic and methanolic extracts of *Agaricus bisporus*, *Cantherellus cibarius*, *Lactarius deliciosus*, and *Pleurotus ostreatus* in RAW 264.7 cells. In addition, Moro et al. (2) reported that the methanolic extracts of *Boletus edulis* inhibited NO production at 500 µg/mL by 10%. Previous studies have reported positive correlations between phenolic compounds and anti-inflammatory effects (3). In addition, Kim et al. (4) reported that cancer is related to inflammatory responses. In this study, the solvent fractions of RNGM were considered to possess excellent potential as materials for preventing related diseases based on their anti-inflammatory effects.

Total polyphenol and flavonoid contents

The total polyphenols in the solvent fractions were determined using the Folin-Ciocalteu's reagent. The total polyphenol content was determined using a linear gallic acid standard curve, and the results are presented in Table 3. The ethyl acetate fraction possessed the highest amount of total polyphenols, followed by the chloroform, water, and n -hexane fractions, which had the lowest [65.33, 41.08, 38.74, and 25.92 mg gallic acid equivalent (GAE)/g, respectively]. Furthermore, the ethyl acetate fraction, which showed the highest yield from RNGM, is considered to have a variety of biological activities. The major factors responsible for the antioxidant activity of phenolic compounds are their chemical structure and redox properties, which allow them to scavenge free radical, chelate transitional metals, and inhibit lipoxygenase (4, 20).

In previous studies, some widely consumed common

Table 3. Total polyphenol, flavonoid, and β -glucan contents of solvent extracts from ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium

Fractions	Total polyphenol (mg GAE/g)	Total flavonoid (mg QE/g)	β -Glucan (%)
n -Hexane	25.92 $\pm$ 1.36 ^c	3.76 $\pm$ 0.82 ^c	0.85 $\pm$ 0.12 ^d
Chloroform	41.08 $\pm$ 0.70 ^b	10.32 $\pm$ 1.02 ^b	1.43 $\pm$ 0.35 ^c
Ethyl acetate	65.33 $\pm$ 0.42 ^a	18.50 $\pm$ 1.29 ^a	13.02 $\pm$ 1.02 ^a
Water	38.74 $\pm$ 2.36 ^b	2.12 $\pm$ 1.57 ^d	6.32 $\pm$ 0.34 ^b

Values are mean $\pm$ SD (n=3).

Means with different letters in the same column (a–d) are significantly different at $P<0.05$.

GAE, gallic acid equivalent; QE, quercetin equivalent.

edible mushrooms in Asia have been found to contain strong antioxidant effects due to their abundant phenolic compound content (18). It is well known that the antioxidant potential of mushroom extracts usually correlates with their phenolic content (3,4). Cheung et al. (3) reported that the antioxidant activities of mushroom extracts were related to their total polyphenol content. In addition, Xiao et al. (21) demonstrated that DPPH and ABTS radical scavenging activities are strongly correlated with antioxidant compounds. The observed high polyphenol content of the fractions could be responsible for the increased antioxidant activities. Based on a previous study (18), the antioxidant capacity of mushrooms is normally associated with phenolic compounds. Therefore, the antioxidant activity of *Ganoderma lucidum* mycelium is also recognized as the effect of contained phenolic compound. These observations led us to infer that the radical scavenging and reducing property of the ethyl acetate fraction might be partially attributed to its phenolic compounds.

Moreover, the results of the total flavonoid content analysis of each RNGM fraction showed that the ethyl

acetate fraction of RNGM had the highest content [18.50 mg quercetin equivalent (QE)/g], followed by the chloroform (10.32 mg QE/g), *n*-hexane (3.76 mg QE/g), and water (2.12 mg QE/g), fractions. Therefore, the total flavonoid content appeared to be related to the antioxidant activities measured for all the fractions because the solvent fraction which has highest flavonoid content showed highest DPPH radical scavenging activity. In addition, the fractions with a neutral polarity contained more total flavonoid content than the other fractions. The result of the phytochemical analysis indicated that considerable quantities of common bioactive components such as terpenoids, alkaloids, phenolics, flavonoids, and saponins were concentrated in the medium polar and polar fractions such as the chloroform, ethyl acetate, and butanol fractions (15). Other study showed that the total polyphenol content was higher in the ethyl acetate fraction than that in the water fractions of *Flammulina velutipes* (3.17~3.50 mg GAE/g) and *Lyophyllum decastes* (1.52~2.92 mg GAE/g) (22). Based on these results, ethyl acetate fractions of RNGM can be considered as potential candidates for the development as functional materials with antioxidant effects.

β-Glucan content

Fibrous structural extracellular matrix within the cell walls of yeast plants are formed by a group of glucose polymers, the β-glucans (8,9). Previous studies have demonstrated that various β-glucans act as immunostimulants that nonspecifically activate cellular and humoral components of the host immune system, which increase the functional activity of macrophages, mononuclear cells, and neutrophils, leading to protection against infectious diseases and cancer (23,24). In this study, the ethyl acetate fraction (13.02%) possessed the highest amount of total β-glucan, followed by the water (6.32%), chloroform (1.43%), and *n*-hexane fractions (0.85%) (Table 3). Previous studies reported that high levels of β-glucans also occur naturally in edible mushrooms such as *Lentinus edodes*, *Grifola frondosa*, *Sparassis crispa* Fr., and *Tremella fuciformis* (24,25). Jung et al. (26) reported that

the β-glucan content of the *Hypsizigus marmoreus* water extract was 9.32%, and Lee and Lee (27) reported the β-glucan content of brown rice cultured with mushroom mycelia extract was 18.4%. In addition, San-Blas and San-Blas (28) demonstrated the generation of high β-glucan in the membrane fraction of the mycelium phases. Beneficial health effects such as antioxidant (29), immunomodulatory, antitumor, antiviral, and anti-inflammatory (24) of the mushroom β-glucan have been documented in previous reviews. Therefore, based on these previous studies, the high antioxidant and anti-inflammatory activity of the ethyl acetate fraction of RNGM might be related to β-glucans.

Isoflavone contents

Soy isoflavones are beneficial in preventing both prostate and breast cancers, reducing the risk of cardiovascular diseases, and alleviating menopausal symptoms (16). Soybeans contain the three isoflavone glucosides: daidzin, genistin, and glycitin as well as their aglycone forms daidzein and genistein, which are biologically active compounds. To measure the isoflavones, each fraction was dissolved in 80% methanol and hydrolyzed with HCl. The isoflavone content of the solvent fractions of RNGM was measured and is shown in Table 4. The results showed that the amount of daidzein and genistein of the methanol extract was found in the ethyl acetate fraction at 19.36 and 11.51 mg/g, respectively. After hydrolysis, the amount of daidzein and genistein was 27.60 and 39.38 mg/g, respectively, in the ethyl acetate fraction. Wang et al. (16) reported that the aglycone content in soybean was less than 2%. Since soybean isoflavones typically contains 80.00~98.37% of biologically inactive glucosides, numerous studies have been conducted to convert glucosides to aglycones by removing the glucose combined with the glucosides. Previous studies reported that the aglycones showed a higher biological activity than the glucosides in the ethyl acetate fraction of soy after digestion (8,9). Jung et al. (30) reported the genistein has an antioxidant effect by scavenging reactive oxygen species. Therefore, the antioxidant effects of the solvent frac-

Table 4. Concentration of isoflavones of solvent fractions of ethanol extract of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium using high-performance liquid chromatography (unit: mg/g)

Samples	Standards	Fractions			
		<i>n</i> -Hexane	Chloroform	Ethyl acetate	Water
Methanol extracts ¹⁾	Daidzein	0.98±0.23	2.53±0.55	19.36±0.12	0.13±0.05
	Genistein	1.03±0.14	2.03±0.32	11.51±0.21	0.72±0.08
	Total	2.01±0.37	4.56±0.87	30.87±0.33	0.85±0.13
Acid hydrolysates ²⁾	Daidzein	5.12±0.15	3.66±0.28	27.60±0.31	0.48±0.19
	Genistein	3.60±0.17	13.93±0.30	39.38±0.35	0.76±0.08
	Total	8.72±0.32	17.96±0.58	66.98±0.66	1.24±0.27

¹⁾Sample was dissolved in 80% methanol.

²⁾Sample was hydrolyzed with 1 N HCl at 100°C for 1 h.

tions of RNGM might be related to isoflavones, which is consistent with the findings of other studies (31).

Based on these results, it is suggested that the ethyl acetate fraction of *Rhynchosia nulubilis* cultivated with *Ganoderma lucidum* mycelium may be a potential natural sources of antioxidant and immunomodulatory and other useful products for nutritional and pharmaceutical applications. Therefore, further studies regarding the components and structure of ethyl acetate fraction are needed.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

REFERENCES

1. Branen AL. 1975. Toxicology and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. *J Am Oil Chem Soc* 52: 59-63.
2. Moro C, Palacios I, Lozano M, D'Arrigo M, Guillaumon E, Villares A, Martínez JA, García-Lafuente A. 2012. Anti-inflammatory activity of methanolic extracts from edible mushrooms in LPS activated RAW 264.7 macrophages. *Food Chem* 130: 350-355.
3. Cheung LM, Cheung PCK, Ooi VEC. 2003. Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chem* 81: 249-255.
4. Kim MY, Seguin P, Ahn JK, Kim JJ, Chun SC, Kim EH, Seo SH, Kang EY, Kim SL, Park YJ, Ro HM, Chung IM. 2008. Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. *J Agric Food Chem* 56: 7265-7270.
5. Sanodiya BS, Thakur GS, Baghel RK, Prasad GB, Bisen PS. 2009. *Ganoderma lucidum*: a potent pharmacological macrofungus. *Curr Pharm Biotechnol* 10: 717-742.
6. Kim GJ, Kim HS, Chung SY. 1992. Effect of varied mushroom on lipid compositions in dietary hypercholesterolemic rats. *J Korean Soc Food Nutr* 21: 131-135.
7. Kang SA, Jang KH, Cho Y, Hong K, Suh JH, Choue R. 2003. Effects of artificial stomach fluid and digestive enzymes on the aglycone isoflavone contents of soybean and black bean (*Rhynchosia molubilis*: Yak-Kong). *Korean J Nutr* 36: 32-39.
8. Pham TT, Shah NP. 2007. Biotransformation of isoflavone glycosides by *Bifidobacterium animalis* in soymilk supplemented with skim milk powder. *J Food Sci* 72: M316-M324.
9. Tsangalis D, Ashton JF, McGill AEJ, Shah NP. 2002. Enzymic transformation of isoflavone phytoestrogens in soymilk by β -glucosidase-producing bifidobacteria. *J Food Sci* 67: 3104-3113.
10. Zhang MW, Park MH, Kim MR. 2016. Study on antioxidant activity and cytotoxicity in cancer cells of extract from waxy sorghum fermented with *Phellinus linteus* mycelium. *J East Asian Soc Diet Life* 26: 418-426.
11. Fakruddin M, Mannan KSB, Mazumdar RM, Afroz H. 2012. Antibacterial, antifungal and antioxidant activities of the ethanol extract of the stem bark of *Clausena heptaphylla*. *BMC Complementary Altern Med* 12: 232-232.
12. Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53: 1841-1856.
13. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. 1982. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. *Anal Biochem* 126: 131-138.
14. Babbar N, Oberoi HS, Uppal DS, Patil RT. 2011. Total phenolic content and antioxidant capacity of extracts obtained from six important fruit residues. *Food Res Int* 44: 391-396.
15. Ishtiaq S, Ahmad M, Hanif U, Akbar S, Mehjabeen, Kamran SH. 2014. Phytochemical and *in vitro* antioxidant evaluation of different fractions of *Amaranthus graecizans* subsp. *silvestris* (Vill.) Brenan. *Asian Pac J Trop Med* 7: S342-S347.
16. Wang G, Kuan SS, Francis OJ, Ware GM, Carman AS. 1990. A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J Agric Food Chem* 38: 185-190.
17. Ham SS, Oh SW, Kim YK, Shin KS, Chang HY, Chung GH. 2003. Antimutagenic and cytotoxic effects of ethanol extract from the *Inonotus obliquus*. *J Korean Soc Food Sci Nutr* 32: 1088-1094.
18. Lee GD, Chang HG, Kim HK. 1997. Antioxidative and nitrite-scavenging activities of edible mushrooms. *Korean J Food Sci Technol* 29: 432-436.
19. Brahmi F, Mechri B, Dabbou S, Dhibi M, Hammami M. 2012. The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. *Ind Crop Prod* 38: 146-152.
20. Reis FS, Martins A, Barros L, Ferreira IC. 2012. Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: a comparative study between *in vivo* and *in vitro* samples. *Food Chem Toxicol* 50: 1201-1207.
21. Xiao Y, Xing G, Rui X, Li W, Chen X, Jiang M, Dong M. 2014. Enhancement of the antioxidant capacity of chickpeas by solid state fermentation with *Cordyceps militaris* SN-18. *J Funct Foods* 10: 210-222.
22. Kim HK, Choi YJ, Jeong SW, Kim KH. 2002. Functional activities of microwave-assisted extracts from *Lyophyllum ulmarium*. *Korean J Food Preserv* 9: 385-390.
23. Shahinian S, Bussey H. 2000. β -1,6-Glucan synthesis in *Saccharomyces cerevisiae*. *Mol Microbiol* 35: 477-489.
24. Babiček K, Čechová I, Simon RR, Harwood M, Cox DJ. 2007. Toxicological assessment of a particulate yeast (1,3/1,6)- β -D-glucan in rats. *Food Chem Toxicol* 45: 1719-1730.
25. Ko YT, Lin YL. 2004. 1,3- β -Glucan quantification by a fluorescence microassay and analysis of its distribution in foods. *J Agric Food Chem* 52: 3313-3318.
26. Jung EB, Jo JH, Cho SM. 2008. Nutritional component and anticancer properties of various extracts from Haesongi mushroom (*Hypsizigus marmoreus*). *J Korean Soc Food Sci Nutr* 37: 1395-1400.
27. Lee HD, Lee KS. 2009. β -Glucan and glucosamine contents in various cereals cultured with mushroom mycelia. *Kor J Mycol* 37: 167-172.
28. San-Blas G, San-Blas F. 1982. Effect of detergents on membrane-associated glucan synthetase from *Paracoccidioides brasiliensis*. *J Bacteriol* 152: 563-566.
29. Deng C, Hu Z, Fu H, Hu M, Xu X, Chen J. 2012. Chemical analysis and antioxidant activity *in vitro* of a β -D-glucan isolated from *Dictyophora indusiata*. *Int J Biol Macromol* 51: 70-75.
30. Jung S, Murphy PA, Sala I. 2008. Isoflavone profiles of soymilk as affected by high-pressure treatments of soymilk and soybeans. *Food Chem* 111: 592-598.
31. Yap YHY, Tan NH, Fung SY, Aziz AA, Tan CS, Ng ST. 2013. Nutrient composition, antioxidant properties, and anti-proliferative activity of *Lignosus rhinocerus* Cooke sclerotium. *J Sci Food Agric* 93: 2945-2952.