韓藥作誌(Korean J. Medicinal Crop Sci.) 27(6) : 419 – 426 (2019)
ISSN(Print) 1225-9306
ISSN(Online) 2288-0186
http://www.medicinalcrop.org
http://dx.doi.org/10.7783/KJMCS.2019.27.6.419

백편두의 항산화 물질 분리 및 동정

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Purification and Identification of Antioxidant Compounds from *Dolichos lablab* L. Seeds

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Received: 2019 August 20
1st Revised: 2019 September 16
2nd Revised: 2019 October 8
3rd Revised: 2019 October 20
Accepted: 2019 October 20

ABSTRACT

Background: This study aimed to identify antioxidant compounds from the seeds of *Dolichos lablab* L. by bioassay-guided isolation and recrystallization.
Methods and Results: The water layer of *D. lablab* L. seed extract inhibits intracellular reactive oxygen species (ROS) expressing the 2',7'-dichlorofluorescein diacetate (DCF-DA), *Cu/Zn* superoxide dismutase (SOD) and catalase genes, as determined by quantitative real-time PCR (qRT-PCR). Two compounds were purified from the water layer of the seeds of *D. lablab* L. using column chromatography and prep-high performance liquid chromatography (HPLC). Using nuclear magnetic resonance (NMR) and electrospray Ionization mass spectrometry (ESI-MS), their chemical structures were identified as 5-[(2-acetyl-2,3-dihydro-1*H*-indazol-1-yl)carbonyl]-4,5-dihydro-3*H*–furan-2-one ($C_{14}H_{14}N_2O_4$) and stachyose.
Conclusions: Two active antioxidant compounds were purified from the seed extract of *D. lablab* L. seed extract and the structures of these compounds were identified as $C_{14}H_{14}O_4N_2$ and stachyose.

Key Words: *Dolichos lablab* L., Chemical Structures, Reactive Oxygen Species Inhibitory Effect, Seed Extract, $C_{14}H_{14}O_4N_2$, Stachyose

INTRODUCTION

Dolichos lablab L. is a leguminous species widespread throughout the tropics. Legumes are important sources of proteins, dietary fiber, carbohydrates, and minerals consumed worldwide. These legumes might be of importance in many zones of developing countries where there is a pressing need for food sources of good protein quality and high energy (Osman, 2007).

The *D. lablab* L. seeds classified by the National Academy of Science, USA as potential source of the protein that have not been explored yet. *D. lablab* is used when there is coldness like a cooling disease, diarrhea and abdominal pain etc. when it is hot weather in summer and plenty of rainy season in oriental medicine.

Phytochemical analysis of *D. lablab* was showed that it contained sugar, phenols, alkaloids, tannins, saponins, terpenoids, steroids, flavonoids, and many other metabolites (Al-Snafi, 2017). *D. lablab* has been studies by some researcher, such as hypolipidemic and hepatoprotective effect from water extract (Ramakrishna *et al*., 2007; Im *et al*., 2016), anti-diabetic activity from methanol (MeOH) extract (Kante and Reddy,

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2013), and anti-microbial activity from n-hexane extract of leaf and flower (Priya and Jenifer, 2014). And dolichin purified from *D. lablab* inhibited Human Immunodeficiency Virus (HIV) reverse transcriptase and α- and β-glucosidase which are glycohydrolase implicated in HIV infection (Ye *et al*., 2000).

Major intracellular reactive oxygen species (ROS) produced are hydroxyl radical, superoxide anion, and hydrogen peroxide (Salvemini *et al*., 2003). Low intracellular ROS is an important mechanism of pathogen killing and also leads to endothelial damage resulting in an increased vascular permeability and cell death (Tiidus, 1998). However, intracellular ROS production plays important role in modulation of release of other inflammatory mediators. This is related mainly to the constitutive expression of NAD(P)H oxidases in various tissues (Bedard and Krause, 2007). ROS produced by this family of enzymes can regulate adhesion molecule expression on endothelium and inflammatory cells, so affecting cell recruitment to the sites of inflammation (Niu *et al*., 1994). They also increase cytokine expression and chemokine (Brzozowski *et al*., 2003). At least part of these effects results from the ability of ROS to stimulate MAPK and NF-kB activity which leads to activation of several transcription factors. It is possible that intracellular ROS may act as second messengers in inflammatory signal transduction (Guzik *et al*., 2003).

In this study, we analyzed antioxidant activity by lipopolysaccharide (LPS)-stimulated RAW264.7 cells in *D. lablab* extract and segregated substances that exhibit antioxidant activity *via* open column, high-performance liquid chromatography (HPLC). And the structure of substances was identified by electrospray ionization-mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). Therefore, we intend to have value as a basic research to discover pharmacology resources with excellent antioxidant effect.

MATERIALS AND METHODS

1. Plant material

The seed of *Dolichos lablab* L. was purchased from commercial, and used for experiment after being dried in the shade.

2. Extraction and fractionation

The dried seed of *D. lablab* (1 ㎏) were ground, and extracted with 10 ℓ water three times at 65℃ (each time for 6 hours). The combined water extract was concentrated *in vacuo* at 40℃ to yield 250 g of residue. The water extract was kept refrigerated at 4℃. And the concentrated extract was resuspended in water and then partitioned successively with diethylether (Et_2O), ethyl acetate (EtOAc), and n-butanol (n-BuOH).

The partitioned water (H_2O) layer was concentrated *in vacuo* at 40℃. The H_2O layer afforded precipitate on standing at a cold chamber. A portion of the extract was subjected to column chromatography [glass column (3 ㎝ × 35 ㎝)] packed with Diaion HP-20 (particle size 250 ㎜, Supelco, Bellefonte, PA, USA) using MeOH gradient (0% to 100%) and water finally as eluent. The 21 fractions were obtained from this initial column chromatographic separation, and then combined to two fractions (A and B) by using thin layer chromatography (TLC) [chloroform : MeOH : water = 60 : 30 : 6 (v/v)]. The active fraction A was rechromatographed on silica gel column (3 ㎝ × 35 ㎝) using the same mixture as eluent to give 16 fractions, and then combined to three factions by using TLC.

3. Purification and identification

Compound I and II from the fraction were purified using an HPLC system, which is consisting of C18 column (4.6 ㎜ × 250 ㎜ I.D, particle size 5 ㎜, SHISEIDO Inc., Osaka, Japan), UV detector 254 ㎚, flow rate 1 ㎖/min, and 1% MeOH as eluent, and the recrystallized with MeOH. Identification of compound I and II were assessed by NMR and ESI-MS.

^{1}H- and ^{13}C-NMR spectra were recorded in deuterium oxide (D_2O) at 500 ㎒ and 100 ㎒, respectively. To confirm the molecular weight of two compounds, we measured the ESI-MS under the same conditions as the NMR. The two compounds confirmed the molecule by comparing the NMR data and the ESI-MS data.

4. Experimental cells

The RAW264.7 cells were purchased from Korea Cell Line Bank (KCLB, Seoul, Korea). They were cultured in Dulbecco's modified eagle's medium (DMEM, Gibco™, Thermo Fisher Scientific, Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS, Seradigm, Radnor, PA, USA), 1% penicillin-streptomycin (Sigma-Aldrich Co., St. Louis, MN, USA) in humidified 5% CO_2 incubator at 37℃. The use of cells was carried out in accordance with the guide for the care and use of cells, Chungbuk National University, Korea.

Table 1. Primer sequences for real-time PCR analysis in RAW264.7 cells.

Primer	Sequences (5′-3′)
Cu/Zn SOD-F	CAGCATGGGTTCCACGTCCA
Cu/Zn SOD-Rev	CACATTGGCCACACCGTCCT
Catalase-F	AAGACAATGTCAACTCAGGTGCGGA
Catalase-Rev	GGCAATGTTCTCACACAGGCGTTT
β-actin-F	CCCACTCCTAAGAGGAGGATG
β-actin-Rev	AGGGAGACCAAAGCCTTCAT

5. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from RAW264.7 cells treated with extracts in the presence or absence of LPS (1 ㎎/㎖) using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Complementary DNA (cDNA) was synthesized using the ReverTra Ace® qPCR RT Master Mix with qDNA Remover (TOYOBO Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. qRT-PCR was performed using the SYBR® Green Real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan) in the CFX96TM Real-time system (Bio-Rad Inc., Herules, CA, USA) with default parameters. Information of primer pairs in qRT-PCR are listed in Table 1.

6. Intracellular ROS measurement

Intracellular oxidative stress was detected using 2',7'-dichlorofluorescein diacetate (DCF-DA) by Warleta *et al.* (2011). RAW264.7 cells treated with or without LPS in the presence of compounds for 24 h were washed twice with Hank's buffered salt solution (HBSS) and incubated with 20 mM of DCF-DA in HBSS for 30 min at 37℃ in a CO_2 incubator. Following wash with PBS, DCF fluorescence was measured at an excitation wave length of 485 ㎚ and emission at 525 ㎚. Since then the reaction was continued for an additional 30 min, and another measurement was performed. The intracellular ROS level was calculated as follows:

$$F = [(A_{30} - A_0)/A_0]] \times 100$$

Where A_0 is the fluorescence before incubation and A_{30} is the fluorescence after incubation for 30 min.

7. Statistical analysis

All the experiments were reiterated at three independent replicates, and the results were presented as means ± standard deviation. Statistical significance was determined by Duncan's Multiple Range Tests (DMRT) using SPSS 12.0 windows version (SPSS Inc., Chicago, IL, USA). Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

1. Identification of compound I and II

Identification of compound I and II were assessed by NMR and ESI-MS. ^{1}H- and ^{13}C-NMR spectra were recorded in D_2O at 500 ㎒ and 100 ㎒, respectively. To confirm the molecular weight of two compounds, we measured the ESI-MS under the same conditions as the NMR. The two compounds confirmed the molecule by comparing the NMR data and the ESI-MS data.

According to the proton NMR result, the peaks of 8 ppm - 9 ppm on proton NMR data were indicated proton of aromatic ring and the area of proton NMR peak is equal to the number of protons. Therefore, the peaks of 8.73, 8.74, 8.75 and 9.03 ppm on proton NMR represent four protons in the aromatic of compound I. Based on the fact that the chemical shift was present and the area value of one unbroken peak displayed in peak of 2.04 ppm are three, it is considered that a methyl substituent is present.

And it was confirmed that it is a methyl substituent attached to acetyl group based on ^{13}C-NMR and 2D-NMR. The following is the carbon NMR result. Based on the number of carbon NMR peaks, we confirmed that a total of 14 carbons are present. The three peaks of 173 ppm - 177 ppm were indicated three carboxyl group. Considering the chemical shift, we confirmed that the six peaks of 127 ppm - 147 ppm was the six carbons of benzene ring. And as a result of further performing 2D-NMR, the 14.51 peak was a methyl substituent attached to acetyl group. The carbon peaks of 26 ppm - 54 ppm were confirmed methylene and methine group.

And as result MS spectrum, the molecular weight of compound I was 274 (Fig. 1). Based on determined 274 molecular weights by MS spectrum and predicted result by NMR, we confirmed that the molecular formula of compound I was identified to 5-[(2-acetyl-2,3-dihydro-1*H*-indazol-1-yl) carbonyl]-4,5-dihydro-3*H*–furan-2-one ($C_{14}H_{14}N_2O_4$) (Fig. 1 and Table 2).

According to the proton NMR result of the compound II, we confirmed anomeric peaks which are specific peaks in the sugar. The proton number 1 of sugar had a characteristic that when it is a cyclic structure, it comes out at ppm higher than other peaks due to the oxygen located on both sides.

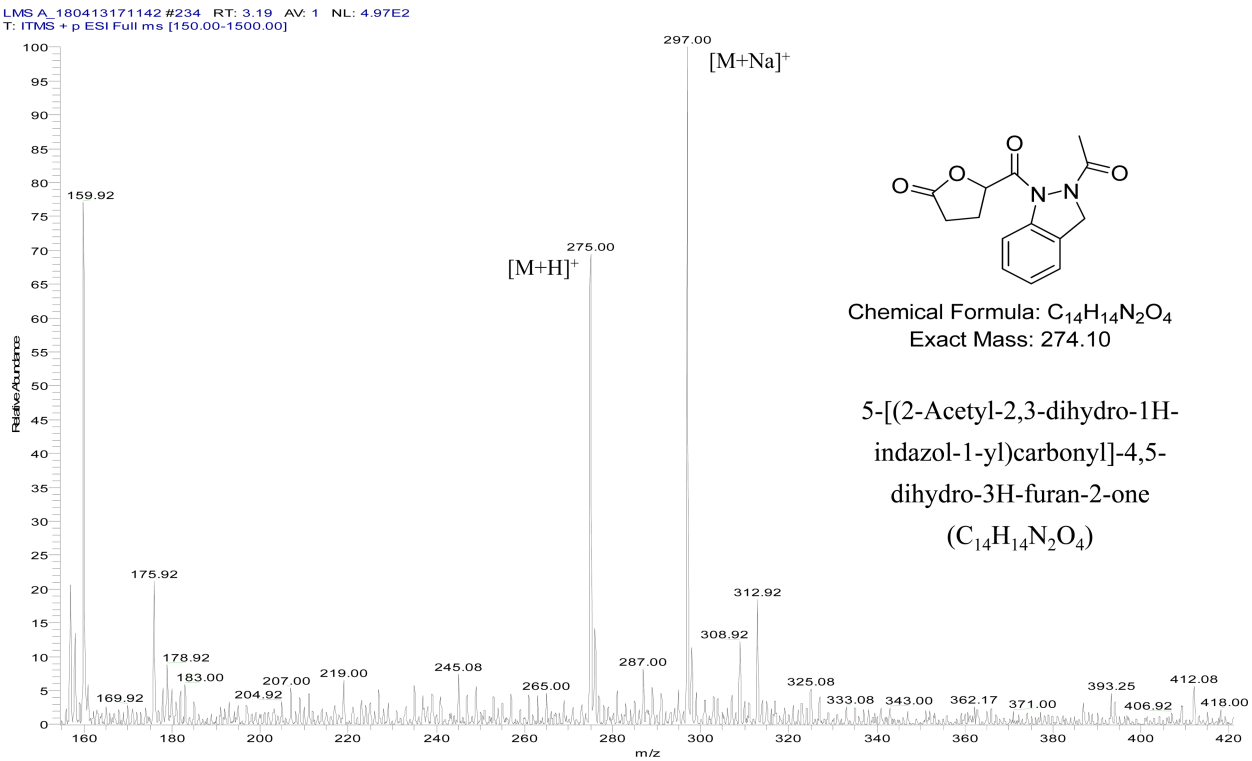

Fig. 1. Chemical structure and ESI-MS data of compound I.

Table 2. ^{1}H and ^{13}C NMR data of compound I.

Carbon NO.	d_H (500 ㎒)	d_C (100 ㎒)
1	2.42 (2H, m)	35.62
2		54.16
3	8.73 (1H, s)	145.87
4	8.74 (1H, s)	144.62
5	8.75 (1H, s)	136.74
6	9.03 (1H, s)	127.50
7		53.96
8		174.23
9	2.68 (1H, d, 6.5)	48.09
10	4.68 (2H, m)	31.57
11	2.49 (2H, m)	26.27
12		167.73
13		177.10
14	2.04 (3H, s)	14.51

Therefore, based on the peaks of 5.35 ppm and 4.91 ppm, the structure of compound II was expected to be a structure with two or more sugar chains. In addition, the area of each peak was 1 : 2, and it was expected that two types of sugar would be connected at a ratio of 1 to 2. Peaks at 3.4 ppm - 4.1 ppm were identified as oxygenated methylene and methine proton, indicating that the peaks are divergent due to complex interactions within the cyclic structure of the sugar. The following is the carbon NMR result. In carbon NMR, anomeric carbons of the sugar were identified. Unlike general methylene and methine, the carbon peaks of 91.98, 97.87, 98.23, and 103.68 at high ppm are thought to be oxygenated anomeric carbon on both sides. In proton NMR, there are three anomeric carbons, whereas there are four anomeric carbons in carbon NMR. Therefore, compound II was identified that fructose without anomeric carbon was presented.

Based on determined 666 molecular weights by MS spectrum (Fig. 2) and predicted result by NMR, we confirmed that compound II was identified to β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1 → 6)-*O*-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranoside (stachyose) (Fig. 2 and Table 3).

2. Antioxidant effect of compound I and II

To confirmed antioxidant activity of two compounds, we confirmed intracellular ROS inhibitory effect through DCF-DA fluorescence and copper/zinc superoxide dismutase (*Cu/Zn*

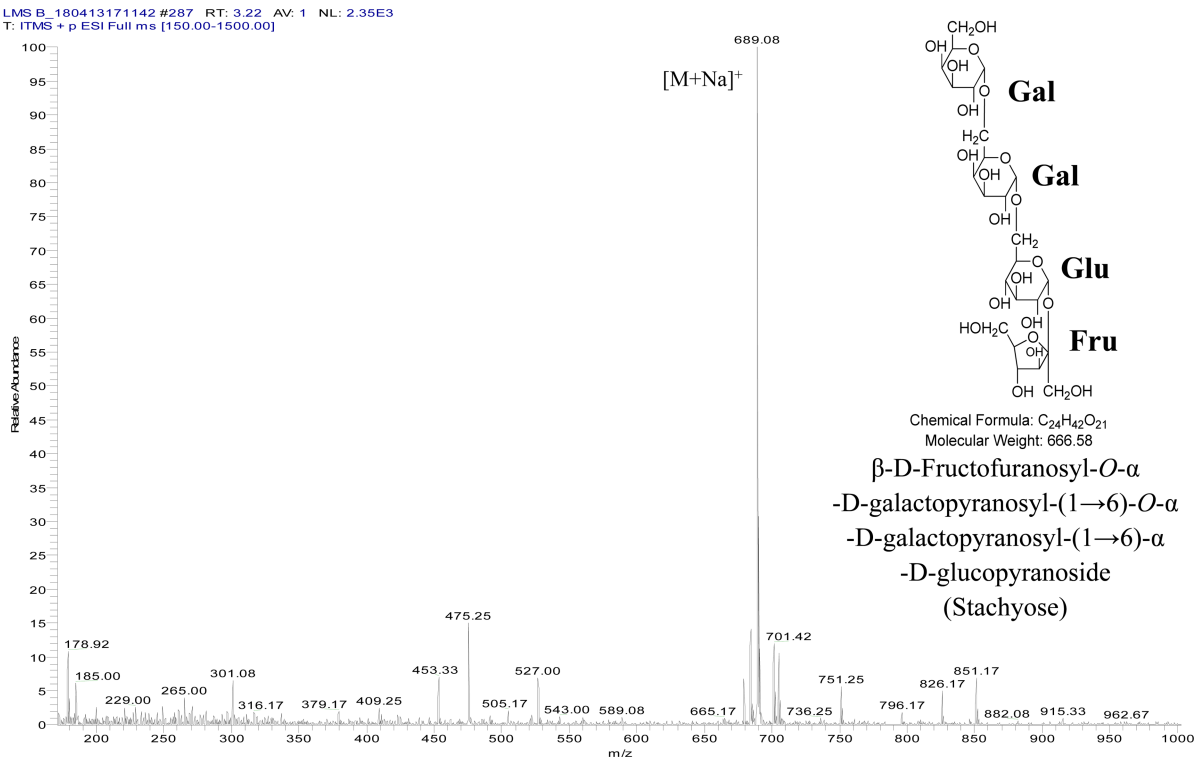

Fig. 2. Chemical structure and ESI-MS data of compound II.

Table 3. ^{1}H and ^{13}C NMR data of compound II.

Carbon NO.	d_H (500 ㎒)	d_C (100 ㎒)
1		62.20
2		103.68
3	3.44 - 4.15	76.11
4	(Sugar parts)	73.81
5		81.22
6		62.34
7	5.35 (1H, d, 3.5)	91.98
8		70.80
9		72.58
10	3.44 - 4.15	68.66
11	(Sugar parts)	71.14
12		65.65
13	4.91 (1H, s)	98.23
14		68.32
15		69.20
16	3.44 - 4.17	69.10
17	(Sugar parts)	68.15
18		66.32
19	4.91 (1H, s)	97.87
20		70.88
21		69.36
22	3.44 - 4.17	69.31
23	(Sugar parts)	69.23
24		61.04

SOD) and catalase genes expression through qRT-PCR. *Cu/Zn SOD* and catalase are enzymes that remove ROS in cells (Michiels *et al*., 1994).

We examined the gene expression levels of catalase and *Cu/Zn SOD* in LPS-treated RAW264.7 cells. The results were shown in Fig. 3. Although the two compounds were not significantly different towards the *Cu/Zn SOD* expression level, the expression level of catalase genes were indicated an increase rate of about 3.5 times in compound I and about 2.4 times in compound II at 50 ㎍/㎖ compared with LPS-not treated cells.

However, the enzyme genes levels of LPS-only treated cells were also increased. It was signified that the stress-affected cells are higher levels of enzyme genes expression than it is not. So, we determined intercellular ROS *via* DCF-DA fluorescence. The result was shown in Fig. 4. It was shown that two compounds inhibited intercellular ROS levels to dose-dependent in LPS-treated RAW264.7 cells.

And through two compounds inhibited intracellular ROS in LPS-not treated RAW264.7 cells, it was confirmed that two compounds did not affect as stress in cells.

Stachyose, β-D-fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside,

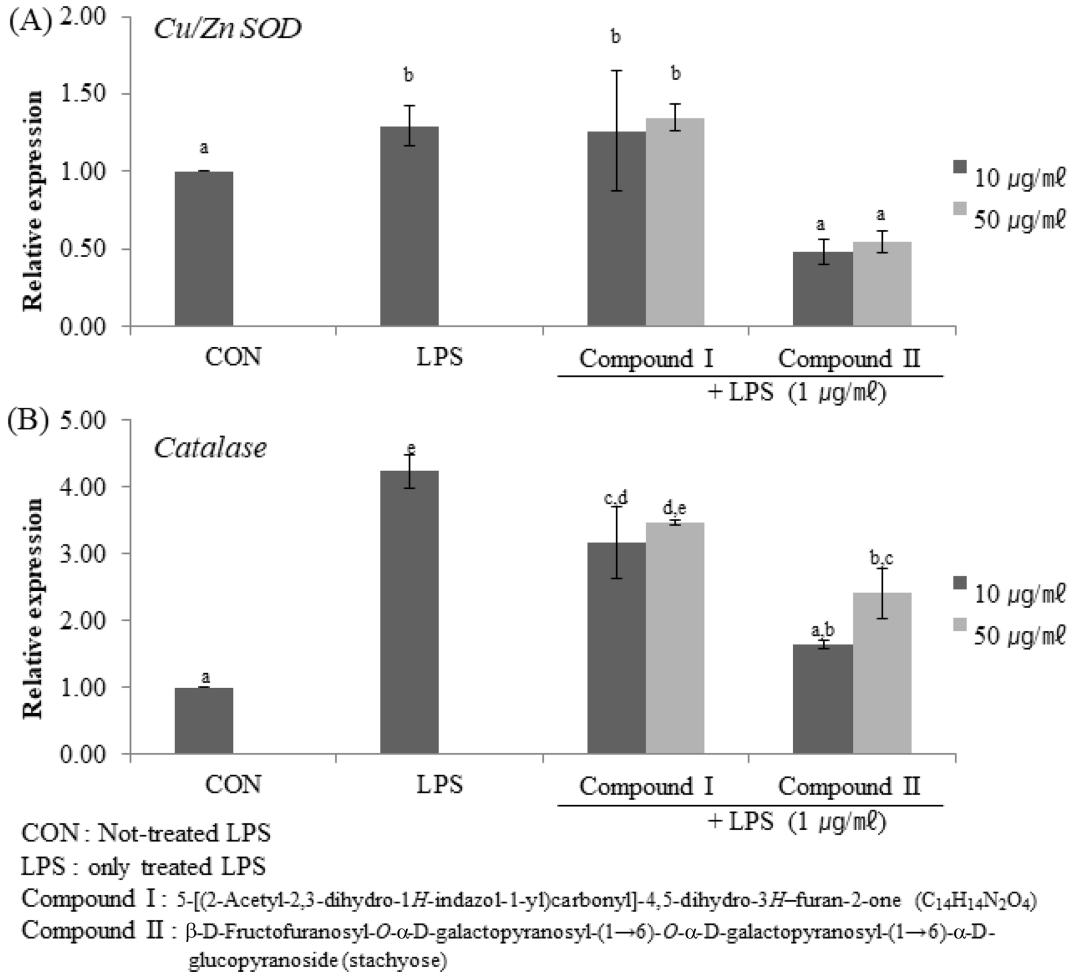

Fig. 3. Effect of Compound I and II on the expression of LPS-induced *SOD* and catalase genes. (A) is the measured value of *Cu/Zn* SOD genes expression through qRT-PCR. (B) is the measured value of catalase genes expression through qRT-PCR. Mean values ± SE from triplicate separated experiments are shown. *Mean within a column followed by same letter are not significant based on the Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

belong to the raffinose family oligosaccharides (RFOs). Physiologically, RFOs in seed may be regarded as storage carbohydrates. They are broken down rapidly during the early stages of germination and may thus provide readily available energy and substrates to support growth (Peterbauer and Richter, 2001). Non-enzymatic antioxidant mechanisms in plant include polyphenols, glutathione, ascorbate, flavonoids, carotenoids, α-tocopherol and sugar-phenols and soluble carbohydrates, such as fructans and RFOs (Stoyanova *et al*., 2011). Also, stachyose indicated to act as an antioxidant in Arabidopsis seed (Nisizawa-Yokoi *et al*., 2008). and inhibited the human epithelial cell line Caco-2 proliferation and induced apoptosis (Huang *et al*., 2015). Therefore, we presumed that extracted starchyose in seed of *D. lablab* was indicated antioxidant activity.

Compound I, 5-[(2-acetyl-2,3-dihydro-1*H*-indazol-1-yl) carbonyl]-4,5-dihydro-3*H*–furan-2-one, form the basic structure by indazole. Indazole was defined by scientist Emil Fisher as a “pyrazole ring fused with the benzene ring”. Indazole nucleus is present in naturally occurring alkaloids and biologically active molecules. In recent years, some of the indazole ring systems are being evaluated as potential drugs for variety of physiological activities with at least few compounds approved for clinical use (Gaikwad *et al*., 2015). A large number of synthetically prepared indazole derivatives have displayed biological and pharmacological properties (Rahman *et al*., 1995). Such as Bendazac is a non-steroidal anti-inflammatory agent, used as an anti-cataract drug (Cerecetto *et al*., 2005). New YC-1 indazole derivative were synthesized and evaluated with hypoxia inducible factor (HIF)-1 transcriptional activity, *in*

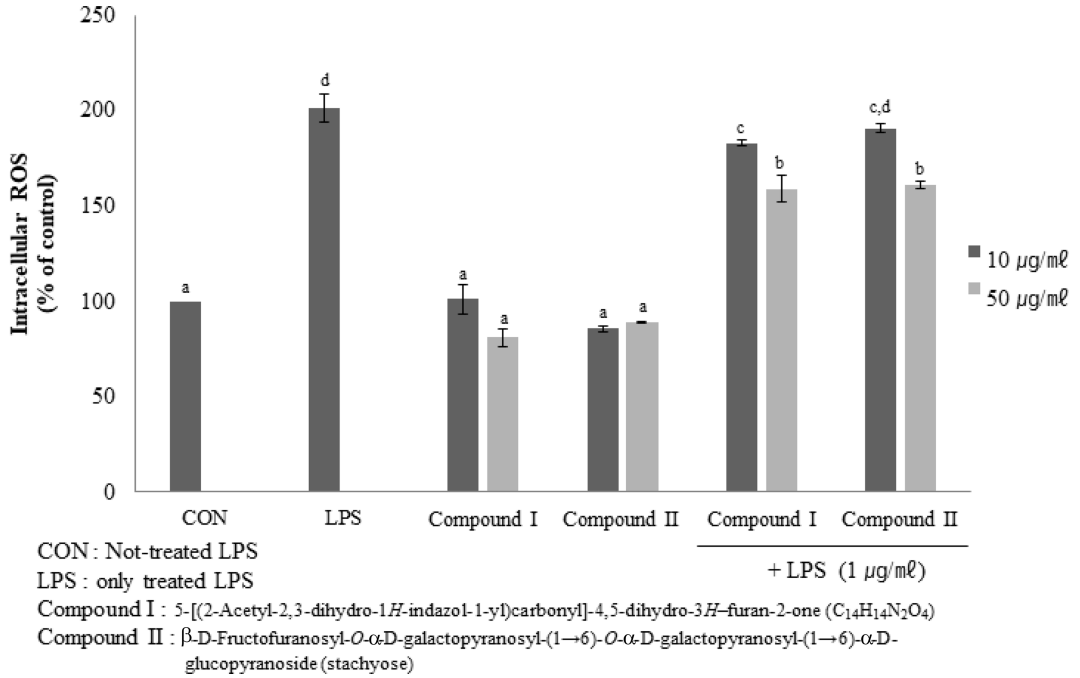

Fig. 4. Effect of Compound I and II on antioxidant activity on LPS-induced RAW264.7 cells. Mean values ± SE from triplicate separated experiments are shown. *Mean within a column followed by same letter are not significant based on the Duncan's Multiple Range Test (DMRT) ($p < 0.05$).

vivo (Takeuchi *et al*., 2012). Thus, we are regarded that extracted compound I in *D. lablab* also shows prominent activity against several diseases.

In conclusion, two antioxidant active compounds were purified from *D. lablab* L. seed extract and the structure was identified as 5-[(2-acetyl-2,3-dihydro-1*H*-indazol-1-yl)carbonyl]-4,5-dihydro-3*H*–furan-2-one and stachyose, respectively.

ACKNOWLEDGEMENTS

This work was carried out with financially supported by the Research Year of Chungbuk National University, Republic of Korea in 2019.

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