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흑남벼 함유 Alkaloid 분리 및 면역효능 연구

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Isolation of Alkaloids with Immune Stimulating Activity from *Oryza sativa* cv. *Heugnambyeo*

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요약. 유색미인 흑남벼(*Oryza sativa* cv. *Heugnambyeo*) 미강의 ethylacetate 가용성 용매분획물로부터 NO 생성을 저해시키는 단일화합물을 순수 분리한 후, HREI-MS 및 2D-NMR 분석을 통해 화학구조를 4-carboethoxy-6-methoxy-2-quinolone (**1**)과 4-carboethoxy-6-hydroxy-2-quinolone (**2**)으로 명명하였다.
주제어: 흑남벼, 유색미, Alkaloid, 면역증진효능, 기능성 쌀

ABSTRACT. We describe the immune stimulatory effects of compounds determined by means of activity-monitored extraction and isolation techniques. As a result, 4-carboethoxy-6-methoxy-2-quinolone (**1**) and 4-carboethoxy-6-hydroxy-2-quinolone (**2**) were isolated from the ethyl acetate-soluble fraction of the *Oryza sativa* cv. *Heugnambyeo* bran, and were determined to exert significant inhibitory effects in macrophage cell line (murine RAW 264.7) and murine splenocytes. The structures were elucidated on the basis of spectroscopic evidence, particularly the results obtained *via* hetero nuclear multiple-bond connectivity and high-resolution MS spectroscopy. Up to date, compound (**1**) was isolated as natural sources for the first time.
Keywords: *Oryza sativa* cv. *Heugnambyeo*, Purple rice, Alkaloid, Immune stimulatory effect, Health-enhancing rice

INTRODUCTION

Bioactive compounds can represent extra-nutritional constituents that are naturally present in small quantities within a food matrix. A variety of chemical entities derived from natural sources have been shown to exert stimulatory effects on diverse immune system functions, including antibody production, resistance to infection, the rejection of malignant cells, *etc*. Select secondary metabolites, originating natural sources and exhibiting biological activity, are considered critical with regard to the promotion of human health. A host of plants and crops have proven to be important sources of a number of secondary metabolites, and it appears reasonable to surmise that additional agents, thus far undiscovered, also exist. With the growth of interest in national health and the expanding health food market, research into the industrial uses of secondary metabolites in crops has also increased.[1] A variety of crops have been shown to possess immune stimulating properties. A great deal of research has, in recent years, focused on the development of new immune stimulatory agents from crops.[2-4] Macrophages are known to play key roles in immune system defense mechanisms, including the following phenomena; pathogens phagocytosis, cytokines production, proteolysis processing, and the presentation of foreign antigens. In addition to the cytokines, nitric oxide (NO) has been identified as a mediator with functions similar to those of these cytokines. NO appears to exert antimicrobial effects, inhibiting the replication of several viruses and parasites.[5] NO also appears to be involved in vasodilatation, nonspecific host defense, ischemia reperfusion injuries, and chronic inflammation.[6]

Rice bran is a rich source of a number of bioactive secondary metabolites, including dietary fiber, phytosteroids, γ-oryzanol, tocopherol and tocotrienols, ferulic acids, and other phenolic compounds. It has been reported that rice bran show cholesterol reduction in plasma and liver, inhibition of platelet aggregation, stimulation of skin microcirculation and prevention of ulcer formation.[7]

Anthocyanin-pigmented rice (*Oryza sativa* cvs. *Heugnambyeo*, *Heugjinjubyeo*, *Hongmi*, *Jakwangdo*, *Kilimheugmi*, *Sanghaehyeolla*, Suwon #405, Suwon #415, Suwon #420, and Suwon #425) was produced by genetic engineering techniques in Korea. This rice has a characteristic dark purple color,

which is attributable to a genomic modification affecting the aleurone layer of the rice grains. This rice variant is used as enriched rice, and is favored for both its taste and its known health benefits in Korea.[8-12]

As a component of our ongoing effort on the characterization of biological activities inherent to highly developed crops, *Oryza sativa* cv. *Heugnambyeo* was selected for this study, as the ethyl acetate-soluble fraction of rice bran was determined to exert a significant inhibitory effect on both a macrophages cell line and on splenocytes. The structures of the isolated and purified compounds were elucidated on the basis of the spectroscopic evidence, particularly the results of hetero nuclear multiple-bond connectivity and high-resolution MS spectroscopy.

In this study, we describe the isolation and identification of two bioactive compounds, 4-carboethoxy-6-methoxy-2-quinolone (**1**) and 4-carboethoxy-6-hydroxy-2-quinolone (**2**), *via* an activity-monitored fractionation and isolation technique,[13] and also demonstrate that compounds **1** and **2** induced a significant inhibition of NO generation (IC_{50} = 20.8 μM and 32.4 μM at a concentration of 20 μg/mL, respectively), in murine macrophages (RAW 264.7), and mouse splenocytes. These results suggest that *Oryza sativa* cv. *Heugnambyeo bran* may prove useful as a naturally immune stimulatory source.

EXPERIMENTAL

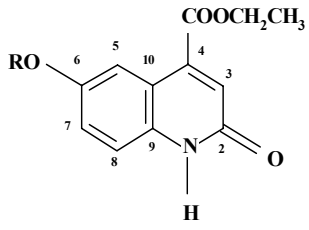
Chemicals. Complete RPMI 1640 medium (Gibco, Invitrogen Co., Carlsbad, California) and FBS (Bio Whittaker, Cambrex Co., Walkersville, Maryland) were used. All other chemicals were purchased from commercial sources and were of the highest purity available.

Instrumental analysis. Melting points (mp) were determined using a Mitamura-Riken melting point apparatus and are uncorrected. Electron impact mass spectrometry (EI-MS) and high resolution EI-MS (HREI-MS) were obtained on a Hewelett Packard Model 5985B Gas chromatography (GC)/MS system and JMS-700 spectrometer, respectively. The Ultraviolet (UV)/Visible (Vis) and Infrared (IR) spectra were recorded on a Hitachi 3100 UV/Vis and JASCO Fourier transform (FT)-IR-5300 spectrophotometer, respectively. A Bruker AMX500 spectrometer was used to record nuclear magnetic resonance (NMR) spectra (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS), and DMSO-d_6 as an internal standard and NMR solvents, respectively. The ^{1}H-^{1}H correlation spectroscopy (COSY), hetero nuclear multiple quantum coherence (HMQC) and hetero nuclear multiple bond correlation (HMBC) experiments were conducted by two-dimensional NMR spectroscopic method. Thin-layer chromatographic (TLC) analysis was performed on silica gel (Kieselgel60 F_{254} plates, 0.25 mm layer thickness; Merck, Darmstadt, Germany), with compounds visualized by spraying with $FeCl_3$, ninhydrin and Dragendorff reagents after developing samples. Silica gel (Merck 60 A, 230 - 400 mesh, ASTM) and Sephadex LH-20 (25 - 100 μm; Pharmacia Fine Chemicals, Piscataway, NJ) were used for open column and vacuum column chromatographic separation.

Plant material. The fully ground *Oryza sativa* cv. *Heugnambyeo* bran was supplied by the National Crop Experiment Station, Rural Development Administration (RDA), Suwon, Gyeonggi-do, Korea, in November 2007. A voucher specimen has been deposited at the RDA.

Extraction, fractionation and isolation of compounds. The dried and ground *Oryza sativa* cv. *Heugnambyeo* bran (3 kg) was extracted with ethyl alcohol (EtOH) for three times for three hours in hot water bath. The combined dark purplish EtOH extracts were partitioned between *n*-hexane and water, with the more polar layer then partitioned with chloroform ($CHCl_3$), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH). The fractions were bio-assayed before additional chromatographic fraction. After then, EtOAc fraction with the desired biological activity was applied for isolation of pure components. The dried EtOAc-soluble fraction (32.7 g) was chromatographed over a silica gel vacuum column using a $CHCl_3$-methanol (MeOH) gradient to give eleven fractions. Fractions 4 and 6 were further chromatographed on a silica gel open column using a $CHCl_3$-MeOH (95:5 to 92:8, v/v), and subfractions 4 - 23 and 6 - 12 were rechromatographed on a Sephadex LH-20 column by elution with MeOH. The each elution on condensation resulted as pale yellow solid materials and was further purified by re-crystallization with highly purified MeOH to give the pure compounds **1** and **2** (*Fig.* 1). Complete identification of isolated compounds made use of varieties of physical and chemical techniques, which included EI-MS and HREI-MS spectrometry, UV/Vis and FT-IR spectrophotometer, and ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC and HMBC spectroscopy.

Isolation of spleen lymphocytes. The spleen was isolated from ICR mice aseptically and minced using slide glasses. Fragments of minced spleen were passed through 100 mesh nylon and a suspension of cells was added two volume of lymphocyte



1: R=CH_3
2: R=H

Fig. 1. Chemical structure of compounds **1** and **2**.

separation medium (Lympholyte-M, Cederlane, Ontario, Canada) and centrifuged at 2,000 × *g* for 20 min at room temperature. After careful sampling of monocytes layer, three cycles of centrifugation, at 1,500 × *g* for 5 min, were done with PBS. Each spleen was fragmented by teasing in 5 mL of ice-cold Hbss, the suspension was transferred to a centrifuge tube and clumps were allowed to a settle by a centrifugation at 1,000 × *g* for 5 min. The supernatant was treated with an ammonium chloride solution using 1.0 mL in 0.1 mL of packed cell volume to dissolve red cells.[14] The cell suspension was centrifuged at 300x*g* for 10 min at 4 °C, washed three times and the number of viable cells was determined microscopically by Trypan blue exclusion test.[15]

Cell culture. Murine macrophage-like cell line (RAW 264.7 cells) and splenocytes were grown in RPMI 1640 containing 2 mM glutamine, 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2.[15]

Preparation of splenocytes. Erythrocytes in splenocytes were disrupted with ACK-lysing buffer (8.29 g/NH_4Cl, 1 g/$KHCO_2$, 37.2 mg/EDTA·2Na). Splenocytes were maintained in RPMI 1640 medium supplemented with 5 mg/mL gentamycin sulfate containing 10% heat-inactivated fetal calf serum.

NO Production. The amount of NO in the cultured medium of macrophages was determined as a nitrite, stable end product of NO. Cultured RAW 264.7 cells were treated with 1% trypsin and washed three times with serum free RPMI 1640 medium (300 × *g*, 5 min). Cells were added to 24 well multi plates at a concentration of 2.0 × 10^5 cells/mL. After culture for 48 hr at 37 °C and 5% CO_2, it was centrifuged for 30 min at 400 × *g*. A 100 μL supernatant was transferred to ELISA titer plate. The 100 μL of Griess reagent (1:1 mixture (v/v) of 1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride in 5% H_3PO_4) was added in each well multi plates, and then mixed well. The mixture was left for 10 min at room temperature and optical density (OD) was measured at 540 nm by a micro plate reader. The nitrite concentration was quantified from the standard curve with $NaNO_3$.

Alkaline phosphatase activity. Colorimetric assays have been used for the assessment of alkaline phosphatase (ALP) activity. The splenocytes were treated with various concentration of EtOH extracts and solvent fractions of *Oryza sativa* cv. *Heugnambyeo* bran in 24 well multi plate and were cultured for 48 hr at a density of 1.0 × 10^6 cells/mL in a 5% CO_2 incubator at 37 °C. The cell suspensions were collected and freeze-thawed. A 100 μL *p*-nitrophenylphosphate 2Na dissolved in 10% diethanolamine-HCl was added to 25 μL of cell lysate. The reaction mixture was incubated at 37°C for 60 min and OD at 405 nm was measured. The stimulation index (SI) of the assay was defined as the ratio of the absorbance signal in control and stimulated cells and calculated as follows; SI = (S-C)/C, where *S* and C represent the absorbance values for the samples and control cells, respectively.

RESULTS AND DISCUSSION

Isolation and structure identification of compounds. The dried and ground *Oryza sativa* cv. *Heugnambyeo* bran was extracted with EtOH and partitioned *via* successive extractions with *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH and H_2O. All solvent

Table 1. ^{1}H-NMR, ^{13}C-NMR and HMBC spectral data of compounds **1** and **2**

Position	δ $^1H^a$ (mult., *J* in Hz)		$^{13}C^b$		HMBC
	1	**2**	**1**	**2**	Correlations
2			160.1(s)[c]	160.1(s)	
3	6.87	6.88	124.0(d)	124.2(d)	C-4, COO
4			116.2(s)	116.2(s)	
5	7.48 (d, *J* = 2.6)	7.49 (d, *J* = 2.6)	109.4(d)	109.4(d)	C-4, 6, 7, 10
6			152.4(s)	152.4(s)	
7	7.09 (dd, *J* = 2.6, 8.9)	7.08 (dd, *J* = 2.6, 8.9)	120.6(d)	120.6(d)	C-6
8	7.24 (d, *J* = 8.9)	7.25 (d, *J* = 8.9)	116.8(d)	116.9(d)	C-4, 5, 6, 7
9			139.3(s)	138.9(s)	
10			132.8(s)	132.8(s)	
COO			165.1(s)	165.9(s)	C-4
CH_2	4.39	4.21	61.7(t)	59.8(t)	COO
CH_3	1.34	1.30	13.8(q)	13.0(q)	COO
OCH_3	3.97		52.7(q)		C-5, COO
NH	11.93	11.95			C-4, 7, CO
OH		9.54			C-5, 6, 7

[a]TMS was used as the internal standard; chemical shifts are shown in the δ scale with *J* values in parenthesis; measured at 500 MHz in DMSO-d_6. [b]Measured at 125 MHz in DMSO-d_6. [c]Multiplicity from DEPT experiments (Θ_y = 45°, 90° and 135°).

fractions were bio-assayed prior to the application of chromatographic separation for the isolation of the bioactive components. The EtOAc-soluble fraction was shown to inhibit 65% of NO generation at a concentration of 20 μg/mL. Therefore, the dried EtOAc-soluble fraction (32.7 g) was chromatographed over a silica gel vacuum column, using a $CHCl_3$-MeOH gradient to generate 11 fractions. Fractions 4 and 6 were then further chromatographed on a silica gel open column using $CHCl_3$-MeOH (95:5 to 92:8, v/v), after which subfractions 4-23 and 6-12 were rechromatographed on a Sephadex LH-20 column by elution with MeOH. Each elution, upon condensation, resulted in the formation of solid materials, which were then additionally purified by re-crystallization with highly purified MeOH, yielding pure compounds **1** (25.6 mg) and **2** (32.7 mg). Compound **1** was obtained as a pale yellow solid, with a molecular weight of 247 based on EI-MS data, and showed a protonated molecular ion peak at *m/z* 248 $[M+H]^+$ and a deprotonated molecular ion peak at *m/z* 246 $[M-H]^-$. These data, together with the data obtained by ^1H NMR and ^{13}C NMR (*Table* 1), indicated a molecular formula of $C_{13}H_{13}NO_4$, which was supported by the findings of HREI-MS in positive ion mode. The compound exhibited UV absorption bands at 240, 282 and 385 nm. These bands remained unaffected by the application of acid, as did the carbonyl absorption band at 1,660 cm^{-1}, and the amide absorption band at 1,625 cm^{-1} in its IR spectrum, there by suggesting the presence of a 2-quinolone skeleton.[16] The ^1H NMR spectrum contained one proton singlet at δ 6.87 (H-3), one triplet and one quartet at δ 4.39 and δ 1.34 for ethyl and methyl protons, respectively, and ABX type signals at three aromatic protons. It was clear that compound **1** had six non protonated carbons, four methane carbons, one ethyl carbon, and two methyl carbon, according to the DEPT and distortionless enhancement by polarization transfer (DEPT; $\Theta_y = 45^o$, 90^o and 135^o) spectra. The HMBC spectrum for long-range correlation with proton and carbon revealed a correlation between the ethyl and methyl group protons and the carboxyl carbon at δ 165.1, which unambiguously indicated the presence of the carboethoxyl moiety at C-4 of the 2-quinolone nucleus. The *meta*-coupled doublet at δ 7.48 (J = 2.6 Hz) in the deshelded position of the aromatic protons was correlated with a quaternary carbon at δ 116.2, assigned to H-4 (*Fig.* 2). The Nuclear Overhauser Enhancements Spectroscopy (NOESY) cross-peak between the *ortho*-coupled doublet at δ 7.24 (J = 8.9 Hz) and the amide proton at δ 11.93 was also observed. These observations showed that the methoxyl group was attached at the C-6 location. Thus, the structure of compound **1** was identified as 4-carboethoxy-6-methoxy-2-quinolone. The occurrence of compound **1** in nature has not been reported previously.

Compound **2** also appeared as a pale yellow solid, with a molecular weight of *m/z* 233 on the basis of the EI-MS data, which showed a protonated molecular ion peak at *m/z* 234 $[M+H]^+$ and a deprotonated molecular ion peak at *m/z* 232 $[M-H]^-$. These data, coupled with the ^1H NMR and ^{13}C NMR data (*Table* 1), indicate a molecular formula of $C_{12}H_{11}NO_4$, which was supported by the HREI-MS spectral data. It showed UV absorption bands at 242, 280 and 382 nm, which were unaffected by the addition of acid, as well as the carbonyl absorption band at 1,658 cm^{-1}, and the amide absorption band at 1,624 cm^{-1} in its IR spectrum, thereby suggesting the presence of a 2-quinolone skeleton. The existence of a phenolic hydroxyl group, as well as the amide group within the molecule, was inferred by the IR bands (1,708 and 3,365 cm^{-1}) and D_2O exchangeable signals located at δ 9.54 and δ 11.95 in the ^1H NMR spectrum. The ^1H NMR spectrum contained a one proton singlet at δ 6.88 (H-3) and ABX type signals at three aromatic protons. It was clear that δ 9.54 and δ 11.95 in the ^1H the compound **2** had six non protonated carbons, four methane carbons, and one methyl carbon, according to the HMQC and DEPT (Θ_y = 45^o, 90^o and 135^o) spectra. The HMBC spectra of compound **2** suggested a structure of 4-carboethoxy-6-hydroxy-2-quinolone, and detailed data are provided in *Table* 1. Compound **2** has been previously isolated from *Oryza sativa* cv. *Heugjinjubyeo*, as a novel antioxidant compound.[11]

Immune stimulating activity. The EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo* bran was investigated, with a focus on its inhibition on NO production and ALP activities in murine peritoneal macrophages and splenocytes, and its potential to enhance immune responses in these cells. Macrophages generate NO, a reactive oxygen species, *via* partial oxygen reduction. The macrophages, exposed to the EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo* bran, generated decreasing quantities of NO in a concentration-dependent manner. At a concentration of 10 μg/mL, the production of nitrite was potentially stimulated by the EtOAc-soluble fraction (*Fig.* 3). However, other solvent fractions, namely the *n*-hexane, $CHCl_3$, and *n*-BuOH-soluble fractions were not stimulated NO production at the concentration of 10 μg/mL. Murine splenocytes

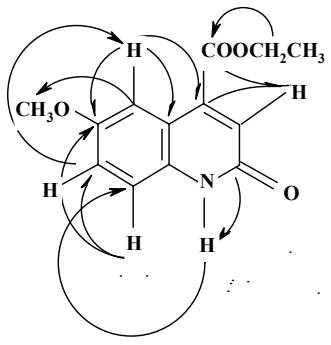


Fig. 2. Selective HMBC correlations of compound **1**.

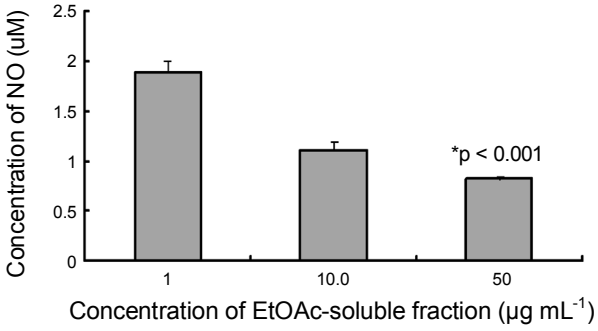


Fig. 3. NO concentration of Raw 264.7 macrophage to the variable concentration of EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo*.

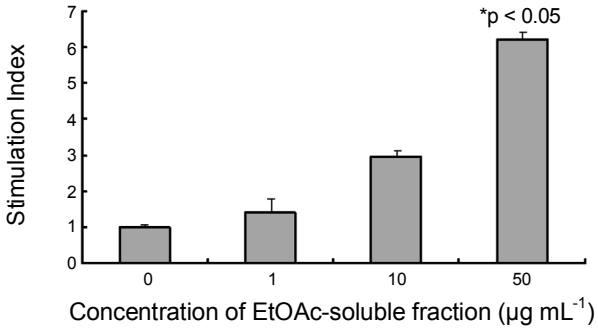


Fig. 4. Alkaline phosphatase activities of splenocytes to the variable concentration of EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo*.

showed correlated activities with the concentration of EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo* bran (*Fig*. 4). There were little differences until 1.0 μg/mL of EtOAc-soluble fraction, however high SI value was observed at the concentration of 10 μg/mL. Hence, we regarded that EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo* bran could stimulate not only nonspecific immune response of macrophages but also humoral immune defense of splenocytes.

In order to characterize the bioactivity of the pure compounds, EtOAc-soluble fraction was chromatographed over a silica gel column using a $CHCl_3$-MeOH gradient, thereby generating 11 sub-fractions, the TLC patterns of which were monitored using a UV lamp with $FeCl_3$, ninhydrin and Dragendorff reaction. Among these sub-fractions, sub-fractions 4 and 6, which evidenced immune stimulatory activity with IC_{50} values of 62.1 and 85.3 μg/mL, were selected for further chromatographic analysis on a silica gel column, *via* elution with $CHCl_3$-MeOH (95:5 to 92:8, v/v), and the resultant sub-fractions 4-23 and 6-12 were then again subjected to chromatography on a Sephadex LH-20 column by elution with MeOH to yield compounds **1** and **2**, respectively. At a concentration of 20 μg/mL, the yields of NO of compounds **1** and **2** were 20.8 and 32.4 μM, respectively. L-NMMA (a NO synthesis-inhibitory agent), used as a positive control, evidenced a significant degree of inhibition, exhibiting a NO yield of 12.7 μM at the equal concentration (*Table* 2). Capsaicin, isolated from the hot pepper, was evaluated with regard to its possible effects on immune status and selected immune responses, including mitogen-induced proliferation, numbers of plaque-forming cells, and total levels of serum immunoglobulin.[17] A great deal of clinical research is currently focused on the identification and analysis of a variety of possible immune modulating agents, including polysaccharides, LPS, and proteins, as they have been shown to exert their influences, principally in terms of cellular rather than humoral immune responses, effecting the restoration of immune competency in impaired hosts without inducing a hyper stimulation in normal hosts.[18] A series of bioactive compounds, including cyanidin, malvidin, cyanidin-3-*O*-*β*-D-glucopyranoside, peinidin-3-*O*-*β*-D-glucopyranoside, anisole, 4-hydroxycinnamic acid, 4,7-dihydroxyvanillic acid, protocatechuic acid methyl ester, ferulic acid and sinapinic acid have been previously isolated from anthocyanin-pigmented rices.[19] The alkaloids, which comprise the largest single class of secondary metabolites, are nitrogenous bases containing one or more nitrogen atoms, which are normally incorporated into a cyclical structure. Alkaloids manifest a variety of dramatic physiological properties, and can be detected quite simply in fresh leaves or fruits *via* tasting alkaloids result in a characteristic bitter taste.[20] The results of this study indicated that alkaloids **1** and **2** isolated from EtOAc-soluble fraction of *Oryza sativa* cv. *Heugnambyeo* bran possessed the ability to stimulate a nonspecific immune response in macrophages, *via* an inhibition of NO generation in the macrophages, as well as a spleen cell activation function. This shows that high quality crops developed *via* genetic engineering can be used, not only to meet increasing production demands, but can also be exploited for their potential bio functional properties.

Table 2. NO concentration of Raw 264.7 macrophage of compounds **1** and **2**

Compounds[a]	Concentration of NO (μM)
1	20.8 ± 0.49
2	32.4 ± 0.60[b]
L-NMMA[c]	12.7 ± 0.31[b]

[a]Each samples were tested at concentration of 20 μg ml^{-1}. [b]Significant level of at $p < 0.05$. [c]Positive control.

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REFERENCES

1. Hyun, J. W.; Chung, H. S. Medicinal plants with cytotoxic and antioxidative activity. In *Recent progress in medicinal plants*, 1st ed.; Govil, J. N., Singh, V. K., Eds.; Studium Press LLC, Texas, **2006,** Vol. 12., pp. 193-202.
2. Yu, K. W.; Kim, Y. S.; Shin, K. S.; Kim, J. M.; Suh, H. J. *Appl. Biochem. Biotechnol.* **2005**, *126*, 35.
3. Wang, J.; Mazza, G. *J. Agric. Food Chem.* **2002**, *50*, 4183.
4. Koh, J. H.; Yu, K. W.; Suh, H. J. *Lett. Appl. Microbiol.* **2002**, *35*, 47.
5. Moncada, S.; Palmer, R. M.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
6. Bredt, D. S.; Snyder, S. H. *Annu. Rev. Biochem.* **1994**, *63*, 175.
7. Ahmed, F.; Platel, K.; Vishwanatha, S.; Puttara, S.; Srinivasan, K. *J. Sci. Food Agric.* **2007**, *87*, 60.
8. Chung, H. S.; Woo, W. S. *J. Nat. Prod.* **2001**, *64*, 1589.
9. Chung, H. S.; Han, H. K.; Ko, J. H.; Shin, J. C. *J. Food Sci. Nutr*. **2001**, *6*, 176.
10. Hyun, J. W.; Chung, H. S. *J. Agric. Food Chem.* **2004**, *52*, 2213.
11. Chung, H. S.; Hyun, J. W. *Food Chem*. **2007**, *104*, 1670.
12. Kang, K. A.; Lee, J. H.; Zhang, R.; Piao, M. J.; Chung, H. S.; Hyun, J. W. *Food Chem*. **2010**, *119*, 1135.
13. Chung, H. S.; Chang, L. C.; Lee, S, K.; Shamon, L. A.; van Breeman, R. G.; Mehta, R. G.; Farnsworth, N. R.; Pezzuto, J. M.; Kinghorn, A. D. *J. Agric. Food Chem.* **1999**, *47*, 36.
14. Daum, J. R.; Shepherd, D. M.; Noelle, R. J. *Int. J. Immunopharmacol.* **1993**, *15*, 383.
15. Kim, H. M.; Oh, G. T.; Hong, D. H.; Hyun, B. H.; Cha, Y. N.; Yoo, B. S.; Han, S. B. *Immunopharmacol.* **1996**, *34*, 39.
16. Gray, A. I. In *Alkaloids and sulfur compounds*. 1st ed.; Waterman, PG, Ed., Academic Press; London, England, **1993**, pp. 271-308.
17. Yu, R.; Park, J. W.; Kurata, T.; Erickson, K. L. *Int. J. Vitam. Nutr. Res.* **1998**, *68*, 114.
18. Nose, M.; Terawaki, K.; Oguri, K.; Ogihara, Y.; Yoshimatsu, K.; Shimomura, K. *Biol. Pharm. Bull.* **1998**, *21*, 1110.
19. Nam, S. H.; Choi, S. P.; Kang, M. Y.; Koh, H. J.; Kozukue, N.; Friedman, M. *Food Chem.* **2006**, *4*, 613.
20. Harborne, J. B. Nitrogen compounds. In *Phytochemical methods*. 2nd ed.; Chapman and Hall, New York, **1981**, pp. 192-194.