Int. J. Indust. Entomol. Vol. 17, No. 2, 2008, pp. 197~200

International Journal of
Industrial Entomology

Antioxidant Activity of *N*-hydroxyethyl Adenosine from *Isaria sinclairii*

Mi Young Ahn*, Jung Eun Heo, Jae-Ha Ryu[1], Hykyoung Jeong, Sang Deok Ji, Hae Chul Park and Ha Sik Sim

Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon 441-100, Korea
[1]College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

(Received 21 November 2008; Accepted 04 December 2008)

The antioxidant activity of *Isaria* (*Paecilomyces*) *sinclairii* was determined by measuring its radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The *n*- BuOH extract of *P. sinclairii* showed strong scavenging activity to DPPH. The anti-oxidant potential of the individual fraction was in the order of ethylacetate > *n*- BuOH > chloroform > *n*-hexane. The *n*-BuOH soluble fraction exhibiting strong anti-oxidant activity was further purified by repeated silica gel column chromatography. *N*-(2-Hydroxyethyl)adenosine (HEA) was isolated as one of the active principles from the *n*-BuOH layer. The *n*-BuOH layer, particularly HEA, did not increase the level of nitric oxide (NO) production in vascular endothelial cells that might be related to vasorelaxation such as the action of viagra. In addition, the vascular endothelial growth factor (VEGF) levels showed little or no increase compared with control group with the treatment of *I. sinclairii*.

Key words: *Isaria sinclairii*, *N*-(2-Hydroxyethyl)adenosine, Antioxidant activity

Introduction

Medicinal mushrooms, which are believed to have a variety of health benefits, have recently attracted the attention of the pharmaceutical industry for the development of nutraceuticals (Wang *et al*., 2003). Cicada Dongchunghacho (a powdered form of *Isaria sinclairii* grown on silkworms) was recently introduced in Korea as a crude drug for the treatment of cancer and diabetes (Ahn *et al*., 2004). Dongchunghacho was also found to possess selective antihypertensive activity in a spontaneously hypertensive rat (SHR) model (Ahn *et al*., 2007a) as well as anti-obesity activity in Zucker fat/fat rats (Ahn *et al*., 2007b). Sensitive high-performance liquid chromatography (HPLC) and tandem mass spectrometry (EI-MS) of the main components of the methanol extract of *I. sinclairii* revealed the presence of adenosine (Ahn *et al*., 2007a). Adenosine may play a role in asthma by enhancing the release of the inflammatory mediator from lung mast cells (Lioyd *et al*., 1998). Moreover, a study using the left atrium of a guinea pig heart in vitro revealed that *N*-(2-hydroxyethyl)adenosine (HEA) from *P. japonica* and some unrevealed Paecilomyces species may also play a role by behaving as Ca^{2+} antagonists and inotropic agents (Furuya *et al*., 1983).

In the case of mulberry leaves, flavonoids have free radical scavenging activity using on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. Of these, quercetin-3-*O*-β-D-glucopyranosyl-(1-> 6)-β-D-glucopyranoside and quercetin were found to possess antioxidant activity (Kim *et al*., 1999). In addition, morusin from mulberry leaves (*Morus alba*) showed weak scavenging activity against superoxide anion radical (Fukai *et al*., 2003). Several nucleosides including adenosine especially erythro-9-(-hydroxy-3-nonyl)adenosine were reported to have a protective effect on rat primary astrocytes against toxicity induced by peroxynitrite and glucose deprivation by preserving the cellular ATP level (Choi *et al*., 2005).

We then analyzed the main components of *I. sinclairii* methanol extract to help understand one of the possible active ingredients with sensitive high-pressure liquid chromatography (HPLC) and tandem mass spectrometry (EI-MS).

This study examined the possible anti-oxidant mecha-

*To whom the correspondence addressed
Department of Agricultural Biology, National Academy of Agricultural Science, 61 Seodun-Dong, Kwonsun-Gu, Suwon 441-100, Korea. Tel: +82-31-290-8577; Fax: +82-31-290-8543; E-mail: amy@rda.go.kr

nism for the action of HEA, which might be associated with endothelial NO dependent vasorelaxation, and the vascular endothelial growth factor (VEGF) level associated with angiogenesis (Lee and Lee, 2005).

This paper reports the antioxidant evaluation of HEA, which was isolated from *I. sinclairii* for its potential to scavenge stable DPPH free radicals and vasculogenic substances from *I. sinclairii* in an attempt to develop a pharmaceutical candidate for treating vasculogenic impotence.

Materials and Methods

Materials

I. sinclairii was collected in Mountain Halla located in Jeju island, South Korea. This fungus endophytically parasitizes dead or living *Cicadae* subspecies. This strain was isolated from conidiospores and cultured in potato dextrose agar (PDA) medium and then sprayed (inoculated) onto silkworms. By evading host defensive mechanisms either during the penetration of the cuticle or upon reaching the hemocoel, *I. sinclairii* proliferated inside larvae to form fruiting bodies. These were cultivated in the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Korea.

Preparation of active principle

The dried *I. sinclairii* (500 g) was soaked and extracted three times with MeOH by ultrasonification for 30 min. The extracts obtained were dried on a rotary evaporation; the residue was suspended in water and sequentially partitioned with hexane, chloroform, ethylacetate, *n*-butanol and H_2O. The *n*-butanol layer showed strong scavenging activity against DPPH radical. Thus, the fraction (3 g) was chromatographed on a silica gel column using CH_2Cl_2-MeOH (gradient) to give compound 1. Each sample (5 mg) was dissolved in 500 µl of phosphate buffered saline (PBS) (final concentration 0.5% ethanol or 0.5% DMSO) as a test solution.

Identification of HEA

MS analysis was performed using a High resolution Tandem Mass spectrometer (Algilent HP 5989B, Palo Alto, CA, USA) using an EI (electron impact)-mode, Electron 70 eV DIP (Direct Inlet Probe) mode in Natural Product Science Research Institute of Seoul National University. All MS data were processed using in the mass databases by the webbook program (NIST, Gaithersburg, MD).

GC-MS analysis

We carried out GC-FAB MS analysis with an Agilent 6890 GC coupled to an Agilent 5973 N mass selective detector, and then the analysis process was regulated with HP 3365 Chem Station software (HP, Palo Alto, CA, USA). A HP 5 MS capillary column (5% PH ME siloxane, 30 m × 0.25 mm, 0.25 µm, USA) was used.

General instruments

NMR spectra were obtained at 600 MHz (^{1}H) and 150 MHz (^{13}C) on a high-resolution spectrometer (Avance 600 FT, Bruker, Germany) using TMS as an internal standard. UV spectra were measured on a JASCO V-550 UV/Vis spectrophotometer and IR spectra using a Jasco FT/IR-3300 spectrophotmeter on KBr plate, and melting points were determined on a Büchi B-540 melting point apparatus.

Identification of N-(2-Hydroxyethyl)adenosine

White powder, FAB-MS m/z: 312 $[M+H]^+$, EI-MS m/z: 311$[M]^+$. UV λ_{max} (DMSO-d_6, log ε): 213 (4.1), 260 (4.2) nm, IR ν_{max} (KBr): 3259 (OH and NH), 1623 (-C=N-) cm^{-1}, ^{1}H-NMR (DMSO-d_6, 600 MHz) δ: 8.43(1H, s, H-8), 8.22 (1H, s, H-2), 7.83 (1H, s, H-10), 5.88 (1H, d, *J*= 6.0 Hz, H-1'), 5.4 ~ 4.8 (4H, br, OH), 4.59 (1H, t, *J*= 5.7 Hz, H-2'), 4.14 (1H, m, H-3'), 3.95 (1H, m, H-4'), 3.57 ~ 3.69 (6H, m, H-5', H-11, H-12); ^{13}C-NMR (DMSO-d_6, 150 MHz) δ: 154.56 (C-6), 152.02 (C-2), 148.42 (C-4), 140.11 (C-8), 120.7 (C-5), 88.10 (C-1'), 86.07 (C-4'), 73.71 (C-2'), 70.76 (C-3'), 61.77 (C-5'), 59.81 (C-12), 42.81 (C-11).

DPPH radical scavenging activity

The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (Blois, 1958). One hundred and sixty microliters of varying sample concentrations (0.25 ~ 160 µg/ml) was added to 40 µl DPPH methanol solution (1.5×10^{-3} M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each fraction and sample was expressed in terms of IC_{50} mg/ml concentration required to inhibit DPPH radical formation by 50% and calculated from the log-dose inhibition curve (Jung *et al*., 2001; Choi *et al*., 2003).

Nitrite assay

The production of nitric oxide (NO) was measured as nitrite accumulated in the culture medium by using a colorimetric reaction with the Griess reagent. In brief, samples were collected 24 hours after the treatment to CPAE (calf pulmonary artery endothelial)/HUVEC cells. The absorbance at 540 nm was measured with a VERSAmax microplate reader (Molecular Devices, Menlo Park, CA, USA). The nitrite concentration was determined from a

sodium nitrite standard curve (Nims *et al.*, 1995).

Cell culture and VEGF measurement

The effects on the production of NO and VEGF was performed on human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA, USA) by using endothelial cell basal medium (EBM)-2 with EGM^{TM}-2 singlequots (Cambrex, Walkersville, USA) at 37°C, 5% CO_2 incubator.

The cytotoxicities of the samples were tested against VEGF cell line using XTT {sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate} kit solution (Boehringer Mannheim), as described previously (Geldof *et al.*, 1999).

Results and Discussion

Identification of N-(2-Hydroxyethyl)adenosine

Compound **1** was purified as a single band on a silica gel TLC showing R_f value of 0.68 with CH_3Cl_3-MeOH (7 : 3) as eluent. The molecular mass was confirmed as 311 from EI-MS and FAB-MS data. The overall spectroscopic data showed characteristic patterns of purine nucleosides including UV λ_{max} at 213, 260 nm with their molecular absorption coefficients and IR bands at 3259 (OH, NH stretching), 1624 cm^{-1} (-C=N- stretching) (Son *et al.*, 1991). The ^{1}H-NMR and HSQC spectra showed that compound **1** has two vinyl hydrogens at δ 8.43 and 8.22, and four hydrogens bound to oxycarbons (δ 88.10, 86.07, 73.71, 70.76) found at δ 5.88~3.95. Another three methylene carbons were found at δ 61.77, 59.81 and 42.81, and those had correlations with overlapped protons at δ 3.57~3.69 in HSQC spectrum. Compound **1** showed characteristic values of proton/carbon chemical shifts corresponding to 5' position of adenosine at δ 3.69 and 61.77, respectively. Compound **1** has extra two methylene groups, one hydroxyl group and one secondary amine proton at δ 7.83 as a singlet peak. This means that one hydroxyethyl goup was bound to primary amine of adenosine. From all these data we identified the structure of compound **1** as N-(2-hydroxyethyl)adenosine. Our spectroscopic data are well matched with the reported data of N-(2-hydroxyethyl)adenosine (Hung *et al.*, 2003; Furuya *et al.*, 1983).

Radical scavenging effect of *I. sinclairii* on the DPPH radical

Table 1 shows the DPPH radical scavenging effect of solvent fractions and the column fractions of *n*-BuOH layer derived from *I. sinclairii*. The *n*-BuOH layer (IC_{50}: 5.08 mg/ml) and the fraction 5-1 (IC_{50}: 11.30 mg/ml), displayed somewhat lower DPPH free radical scavenging activity, as shown in Table 1.

Table 1. Anti-oxidant activity of IS extracts on DPPH

IS Extract	DPPH[a]	Extract	Fraction	DPPH[a]
$CHCl_3$	5.22	*n*-BuOH	3-3	0.78
EtOAC	1.14		5-1	11.30
			7	8.11
n-BuOH	5.08		7-4	0.37
H_2O	17.07		8	18.67
L-Ascorbic acid	2.28			
Pyrogallol	0.24			

DPPH[a]: DPPH free radical scavenging activity (IC_{50}: mg/ml).

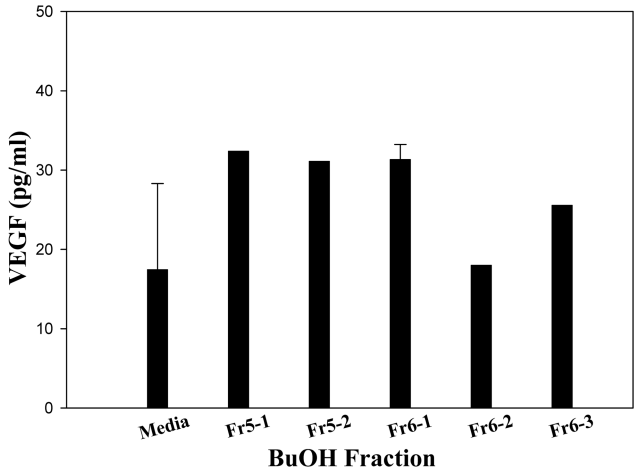

Fig. 1. *N*-BuOH column fractions on VEGF levels in HUVEC cell culture system.

Butanol fractions having *N*-(2-Hydroxyethyl)adenosine from the *Isaria sinclairii* (5 mg/ml) appeared the unremarkable binding of mouse VEGF polyclonal antibody.

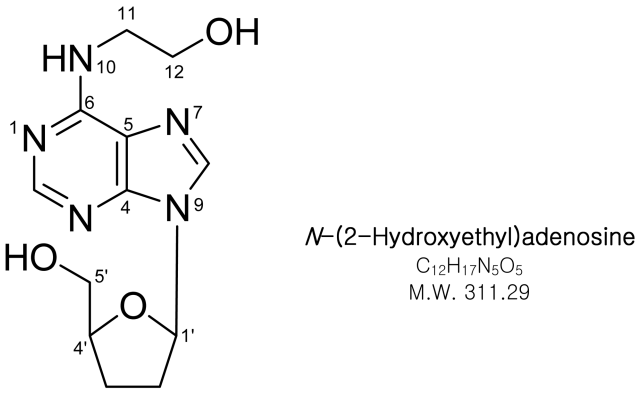

Fig. 2. Structures of *N*-(2-Hydroxyethyl)adenosine.

Effects of VEGF on the *n*-BuOH layer of *I. sinclairii*

The *n*-BuOH column fraction showed strong scavenging activity against the DPPH radical. Furthermore, the

VEGF levels increased slightly compared with the control by the treatment of *I. sinclairii*, as shown in Fig. 1.

In addition, the *n*-BuOH layer did not increase the level of nitric oxide (NO) production in vascular endothelial cells, which might contribute to the vasorelaxation of blood vessels *in vivo* leading to decrease in blood pressure. Nitric oxide enhances angiogenesis via the synthesis of vascular endothelial growth factor and cGMP after stroke in rat (Zhang *et al.*, 2003). In this study, there was little effet on *I. sinclairii* on the VEGF production in the HUVEC endothelial cells, which suggests that this has virtually no relationship with angiogenesis associated with the cancer progression state (Fig. 1).

References

Ahn, M. Y., S. D. Jee, J. Y. Kim, J. W. Han, Y. K. Lee, Y. W. Lee, K. S. Ryu, B. M. Lee, N. J. Jung and S. N. Kim (2004) Thirteen-week repeated oral toxicity study of *Paecilomyces sinclairii* in Sprague-Dawely rats. *J. Toxicol. Pub. Health* **20**, 339-348.

Ahn, M. Y., Y. S. Jung, S. D. Jee, C. S. Kim, S. H. Lee, C. H. Moon, S. I. Cho, B. M. Lee and K. S. Ryu (2007a) Antihypertensive effect of the Dongchunhacho, *Isaria sinclairii,* in the spontaneously hypertensive rats. *Arch. Pharm. Res.* **30**, 493-501.

Ahn, M. Y., S. D. Jee and B. M. Lee (2007b) Anti-obesity effects of *Isaria sinclairii* by repeated oral treatment in obese Zucker rats over a 4-month period. *J. Toxicol. Environ. Health* **70**, 1395-1401.

Blois, M. S. (1958) Antioxidant determinations by the use of stable free radicals. *Nature* **26**, 1199-1200.

Choi, J. W., B. K. Yoo, M. K. Ryu, M. S. Choi, G. H. Park and K. H. Ko (2005) Adenosine and purine prevent the disruption of mitochondrial transmembrane potential by peroxynitrite in rat primary asrocytes, *Arch. Pharm. Res.* **28**, 810-815.

Furuya, T., M. Hirotani and M. Matsuzuwa (1983) N^6-(2-hydroxyethyl)adenosine, a biologically active compound from cultured mycelia of cordyceps and Isaria species. *Pytochemistry* **22**, 2509-2512.

Fukai, T., K. Satoh, T. Nomura and H. Sakagami (2003) Antinephritis and radical scavenging activity of preylflavonoids. *Fitoterapia* **74**, 720-724.

Geldof, A. A., S. C. Mastbergen, R. E. Henrar and G. T. Faircloth (1999) Cytotoxicity and neurocytotoxicity of new marine anticancer agents evaluated using *in vitro* assays. *Cancer Chemother. Pharmacol.* **44**, 312-318.

Hung, L. F., Y. Z. Liang, F. Q. Guo, Z. F. Zhou and B. M. Cheng (2003) Simultaneous separation and determination of active components in *Cordyceps sinensis* and *Coryceps militarris* by LC/ESI-MS. *J. Pharm. Biomed. Anal.* **33**, 1155-1162.

Jung, M. J., H. Y. Chung and J. S. Choi (2001) Antioxidant activity of roasted deffated Perilla seed. *Nat. Prod. Sci.* **7**, 72-75.

Kim, S. Y., J. J. Gao, W. C. Lee, K. S. Ryu, K. R. Lee and Y. C. Kim (1999) Antioxidative flavonoids from the leaves of *Morus alba*. *Arch. Pharm. Res.* **22**, 81-85.

Lee, S. J. and H. K. Lee (2005) Sanguiin H-6 blocks endothelial cell growth through inhibition of VEGF binding to WEGF receptor. *Arch Phram Res.* **11**, 1270-1274.

Lioyd, H. G. E., L. Ross, K. M. Li and R. I. Ludowyke (1998) Evidence that IgE receptor stimulation increases adenosine release from rat basophilic leukaemia (RBL-2H3) cells. *Pulm. Pharmacol.Ther.* **11**, 41-46.

Nims, R. W., J. F. Darbyshire and J. E. Saavedra (1995) Colorimetric methods for the determination of nitric oxide concentration in neutral aqueous solutions. *Methods* **7**, 48-54.

Son, K. H., J. C. Do and S. S. Kang (1991) Isolation of adenosine from Rhizomes of *Polygonatum sibiricum*. *Arch. Pharm. Res.*, **14**, 193-194.

Wang, G. J., H. W. Tseng, C. J. Chou, T. H. Tsai, C. T. Chen and M. K. Lu (2003) The vasorelaxation of *Antrodia camphorata* mycelia: Involvement of endothelial Ca^{2+}-NO-cGMP pathway. *Life Science* **73**, 2767-2783.