Antioxidant Activity of \(N\)-hydroxyethyl Adenosine from *Isaria sinclairii*

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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 (Lloyd 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-\(\beta\)-D-glucopyranosyl-(1-> 6)-\(\beta\)-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-
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 Cicadidae 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, the residue was suspended in water and sequentially partitioned with hexane, chloroform, ethylacetate, and H₂O. The n-butanol layer showed strong scavenging activity against DPPH radical. Thus, the fraction (5 g) was chromatographed on a silica gel column using CH₂Cl₂-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 (Agilent 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 (¹H) and 150 MHz (¹³C) 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 spectrophotometer 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 λmax (DMSO-d₆, log ε): 213 (4.1), 260 (4.2) nm, IR νmax (KBr): 3259 (OH and NH), 1623 (-C=N-). ¹H-NMR (DMSO-d₆, 600 MHz) δ: 6.23 (1H, s, H-11), 8.22 (1H, s, H-2), 7.83 (1H, s, H-10), 8.07 (1H, d, J=6.4 Hz, H-3), 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), 3.69 (6H, m, H-5', H-11, H-12); ¹³C-NMR (DMSO-d₆, 150 MHz) δ: 154.56 (C-6), 152.02 (C-2), 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 × 10⁻⁵ 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 IC₅₀ 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., 2005).

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 were performed on human umbilical vein endothelial cells (HUVEC) (ATCC, Manassas, VA, USA) by using endothelial cell basal medium (EBM)-2 with EGM™-2 single quot (Cambrex, Walkersville, USA) at 37°C, 5% CO₂ incubator.

The cytotoxicities of the samples were tested against VEGF cell line using XTT [sodium 3'-[1-(phenylamino-carbonyl)-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 R₅ value of 0.68 with CH₃Cl₂-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 λₘₐₓ 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 ¹H-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 group 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 (IC₅₀: 5.08 mg/ml) and the fraction 5-1 (IC₅₀: 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

<table>
<thead>
<tr>
<th>IS Extract</th>
<th>DPPH ⁰ Extract</th>
<th>Fraction</th>
<th>DPPH ⁰</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHCl₃</td>
<td>5.22</td>
<td>n-BuOH</td>
<td>3-3</td>
</tr>
<tr>
<td>EtOAC</td>
<td>1.14</td>
<td>5-1</td>
<td>7</td>
</tr>
<tr>
<td>n-BuOH</td>
<td>5.08</td>
<td>7-4</td>
<td>0.37</td>
</tr>
<tr>
<td>H₂O</td>
<td>17.07</td>
<td>8</td>
<td>18.67</td>
</tr>
<tr>
<td>L-Ascorbic acid</td>
<td>2.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrogallol</td>
<td>0.24</td>
<td></td>
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</tr>
</tbody>
</table>

DPPH: DPPH free radical scavenging activity (IC₅₀ 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.**

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 effect 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**


