Natural Product Sciences
18(3) : 147-152 (2012)

Inhibition of Nitric Oxide Production, iNOS and COX-2 Expression of Ergosterol Derivatives from *Phellinus pini*

Yun Jung Hong, A Reum Jang, Hyun Jin Jang, and Ki Sook Yang*

College of Pharmacy, Sookmyung Women's University, Seoul 140-742, Korea

Abstract – Ergosta-4,6,8(14),22-tetraen-3-one (**1**), ergosta-7,24(28)-dien-3-ol (**2**), and 5,8-epidioxyergosta-6,22-dien-3-ol(**3**) were isolated from the fruit body of *Phellinus pini*. Their structures were based on spectroscopic methods including IR, MS, and NMR (1D and 2D). These compounds were screened for their ability to inhibit nitric oxide (NO) production in LPS-activated RAW 264.7 cells. Compounds **1**, **2**, and **3** reduced NO production in the assay with IC_{50} values of 29.7 μM (**1**), 15.1 μM (**2**), and 18.4 μM (**3**) respectively. They also suppressed the expression of protein and m-RNA of iNOS and COX-2 in a dose dependent manner by western blot analysis and RT-PCR experiment in LPS-activated microglial cells.
Keywords – *Phellinus pini,* RAW 264.7 cells, Nitric Oxide, iNOS, COX-2

Introduction

Phellinus pini Ames (Hymenocaetaceae) is a white-rot fungus that frutifies over the stems of Pinaceae, Cupressaceae etc. This mushroom has been known for its immunomodulating (Jeong *et al*., 2004), hypolipidemic (Yang *et al*., 2002), and anti-inflammatory (Jang and Yang, 2011) activities. The antitumor activities and immunostimulating activities of the polysaccharide fraction of this mushroom have been investigated (Ikegawa *et al*., 1968). As compounds, ergosterol, p-hydroxybenzoic acid, and vanillic acid were isolated from the fruit body of *Phellinus pini* (Epimenko and Ageenkova, 1965), 2-farnesyl-5-methylbenzoquinone, 2,4,6-triphenylhex-1-ene were isolated from the fungus of *Phellinus pini* and had a antifungal activities (Ayer *et al*., 1996). Polysaccharide from *Phellinus pini* had a antifungal activity (Lee *et al*., 2010). As a part of our continuing search for new anti-inflammatory activities from natural sources, we isolated active compounds from the fruit body of *Phellinus pini* and evaluated the inhibitory activities on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated macrophage RAW 264.7 cells and expressions of protein and m-RNA of iNOS and COX-2.

Experimental

General experimental procedures – IR spectra were obtained using a Jasco FT-IR 430 spectrophotometer. GC-Mass spectra were measured with a JEOL JMS-AX505WA HP5890 mass spectrometer. Both ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 500 NMR spectrometer. Silica gel (70-230 mesh, Merck Co.) was used for column chromatography and silica gel $60F_{254}$ (Merck) for TLC.

Plant material – In this study we used the fruit body of *Phellinu pini* imported by Beakjae, in the Kyeongdong trading center. A voucher specimen was deposited at the herbarium in the College of Pharmacy, Sookmyung Women's University.

Extraction and isolation – The fruit bodies (5 kg) of the mushroom were ground and extracted 3 times with methanol for 4 h, yielding, after removal of the solvent under vacuum, 297 g of dried extract. This methanol extract was partitioned with hexane, dichloromethane, ethyl acetate, butanol and water. The hexane fraction (12 g) was chromatographed over silica gel with solvent gradient elution (hexane:EtOAc, 10 : 1 → 5 : 1) and eight fractions were collected. Fraction 5 (420 mg) was repeatedly chromatographed over silica gel using CH_2Cl_2-MeOH (50 : 1) as the eluant and we obtained subfractions. Fraction 5 - 4 (80 mg) was repeatedly chromatographed over silica gel using benzene:acetone (30 : 1) as eluant and we isolated compound **1** (40 mg) from fraction 5-4-1. Fraction 7 (500 mg) was chromatographed over silica gel using hexane:ethylacetate (2 : 1) as the eluant and we isolated compound **2** (30 mg) from

*Author for correspondence
Tel: +82-2-710-9578; E-mail: ksyang@sookmyung.ac.kr

fraction 7-3. Fraction 8 (550 mg) was repeatedly chromatographed over silica gel using hexane:acetone (2 : 1) as eluant and we obtained subfraction 8-5 (175 mg). It was repeatedly chromatographed over silica gel using hexane-ethylacetate-isopropanol (1 : 1 : 0.1) as eluant and we isolated compound **3** (38 mg) from fraction 8-5-5.

Ergosta-4,6,8(14),22-tetraen-3-one (1) (ETO) – Yellow plate, $C_{28}H_{40}O$, IR ν_{max} ($CDCl_3$, cm^{-1}): 2956 (aliphatic C-H), 1664 (C = O), 1587 (C = C); EI-MS (*m/z*): 392 $[M]^+$, 377 $[M\text{-}CH_3]^+$, 349 $[M\text{-}C_3H_7]^+$, 267 $[M\text{-}C_9H_{17}]^+$; ^{1}H-NMR (600 MHz, $CDCl_3$): δ 0.76 (3H, d, *J* = 6.8Hz, CH_3-26), 0.77 (3H, d, *J* = 6.8Hz, CH_3-27), 0.86 (3H, d, *J* = 6.8 Hz, CH_3-28), 0.89 (3H, s, CH_3-18), 0.92 (3H, s, CH_3-19), 0.99 (3H, d, *J* = 6.7 Hz, CH_3-21), 5.13 (1H, d,d, *J* = 8.2Hz, 15.3Hz, H-23), 5.19 (1H, d,d, *J* = 7.5Hz, 15.3Hz, H-22), 5.66 (1H, s, H-4), 5.95 (1H, d, *J* = 9.5 Hz, H-6), 6.54 (1H, d, *J* = 9.5 Hz, H-7); ^{13}C-NMR (150 MHz, $CDCl_3$): δ 35.53 (C-1), 34.08 (C-2), 199.51 (C-3), 122.93 (C-4), 156.17 (C-5), 124.41 (C-6), 134.00 (C-7), 156.08 (C-8), 44.26 (C-9), 36.71 (C-10), 18.94 (C-11), 34.08 (C-12), 43.94 (C-13), 164.39 (C-14), 25.33 (C-15), 27.70 (C-16), 55.63 (C-17), 18.91 (C-18), 16.61 (C-19) 39.27 (C-20), 21.19 (C-21), 132.47(C-22), 134.96 (C-23), 42.83 (C-24), 33.04 (C-25), 19.96 (C-26), 19.63 (C-27), 17.61 (C-28).

Ergosta-7,24(28)-dien-3-ol (2) (EDO) – Colorless needle, $C_{28}H_{46}O$, IR ν_{max} ($CDCl_3$, cm^{-1}): 3393 (O-H), 2955 (aliphatic C-H), 1715 (C = C), 1039 (C-O); EI-MS (*m/z*): 398 $[M]^+$, 383 $[M\text{-}CH_3]^+$, 314 $[M\text{-}C_6H_{12}]^+$, 271 $[M\text{-}C_9H_{17}\text{-}H_2]^+$, 255 $[M\text{-}C_9H_{17}\text{-}H_2O]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.54 (3H, 18-CH_3), 0.79 (3H, 19-CH_3) 0.85 (3H, d, *J* = 6.4Hz 21-CH_3), 1.02 (3H, d, *J* = 7.0Hz 27-CH_3), 1.03 (3H, d, *J* = 7.0Hz 26-CH_3), 3.61 (1H, s, 3-H), 5.18 (1H, m, 7-H), 4.66(1H, s, 28-H), 4.71 (1H, s, 28-H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 36.38 (C-1), 37.34 (C-2), 71.29 (C-3), 38.09 (C-4), 40.46 (C-5), 38.09 (C-6), 117.68 (C-7), 139.74 (C-8), 49.65 (C-9), 34.85 (C-10), 19.04 (C-11), 31.28 (C-12), 40.46 (C-13), 39.77 (C-14), 21.75 (C-15), 23.16 (C-16), 29.85 (C-17), 12.05 (C-18), 13.23 (C-19), 56.22 (C-20), 19.04 (C-21), 34.85 (C-22), 39.77 (C-23), 157.02 (C-24), 55.24 (C-25), 22.06 (C-26), 22.19 (C-27), 106.17 (C-28).

5,8-Epidioxyergosta-6,22-dien-3-ol (3) (EPO) – White needles, $C_{28}H_{44}O_3$, IR ν_{max} ($CDCl_3$, cm^{-1}): 3394 (O-H), 2955 (aliphatic C-H), 1042 (C-O); EI-MS (*m/z*): 428 $[M]^+$, 410 $[M\text{-}H_2O]^+$, 396 $[M\text{-}O_2]^+$, 363 $[M\text{-}O_2\text{-}H_2O\text{-}CH_3]^+$, 271 $[M\text{-}C_9H_{17}\text{-}O_2]^+$; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 0.81 (3H, s, 18-CH_3), 0.82 (3H, d, *J* = 7.0Hz, 27-CH_3), 0.84 (3H, d, *J* = 6.9Hz, 26-CH_3), 0.88 (3H, s, 19-CH_3), 0.90 (3H, d, *J* = 6.8Hz, 28-CH_3), 0.99 (3H, d, *J* = 6.6Hz, 21-CH_3), 3.94 (1H, m, 3-H), 5.15 (1H, dd, *J* = 8.4, 15.3Hz, 23-H), 5.23 (1H, dd, *J* = 7.5, 15.3Hz, 22-H), 6.23 (1H, d, *J* = 8.5Hz, 6-H), 6.48 (1H, d, *J* = 8.5Hz, 7-H); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 34.90 (C-1), 30.26 (C-2), 66.57 (C-3), 37.10 (C-4), 79.61 (C-5), 130.90 (C-6), 135.63 (C-7), 82.36 (C-8), 51.30 (C-9), 37.16 (C-10), 21.07 (C-11), 39.54 (C-12), 44.75 (C-13), 51.88 (C-14), 23.59 (C-15), 28.81 (C-16), 56.40 (C-17), 13.06 (C-18), 18.35 (C-19), 39.89 (C-20), 20.82 (C-21), 135.39 (C-22), 132.49 (C-23), 42.96 (C-24), 33.25 (C-25), 20.13 (C-26), 19.82 (C-27), 17.75 (C-28).

Cell culture – A mouse macrophage cell line (RAW 264.7 cells) was purchased from the Korean Cell Line Bank. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 ug/mL streptomycin and 0.25 ug/mL amphotericin. Cells were maintained at 37 ºC under 5% CO_2 in a fully humidified atmosphere.

MTT assay for viability – The RAW 264.7 cells were seeded at 1.5×10^5 cells/mL in 48-well plates and incubated for 2 h, then treated with varying concentrations of compounds **1**, **2**, and **3**. The cells were incubated for 24 h and then moved to fresh medium containing 0.5 mg/mL MTT. The incubation continued for an additional 4 h at 37 ºC. The medium was removed and the MTT-formazan produced was dissolved in 200 uL DMSO. The extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance of the DMSO solution at 570 nm using an ELISA reader (Mosmann, 1983). Cytotoxicity was calculated as the reduction in cell viability.

Measurement of NO production – RAW 264.7 cells were plated at a density of 1.5×10^5 cells/mL in 48-well cell culture plates containing 400 uL of culture medium. Cells were incubated for 24 h. The cells were then treated with 1 ug/mL LPS and various concentrations of test samples for 20 h. Nitrite was measured by adding 150 uL of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 uL of culture medium. Absorbance at 570 nm was measured using a microplate reader, and the nitrite concentration was calculated by comparison with the absorbance of standard solutions of sodium nitrite, with L-NMMA used as a positive control.

Western blot analysis – RAW 264.7 cells were seeded at 5×10^5 cells/mL in 60mm cell culture plates containing 4 mL of culture medium. Cells were incubated for 24 h. and then treated with 1 ug/mL LPS with various concentrations of test samples for 20 h. The cells were rinsed with phosphate buffer saline and lysed with iced-lysis buffer (Pro-Prep™, Intron Biothech., Korea) and

Table 1. Sequences of primers

Gene	Primer sequence
iNOS	
Forward	CCC TTC CGA AGT TTC TGG CAG CAG C
Reverse	GGC TGT CAG AGC CTC GTG GCT TTG G
COX-2	
Forward	CAC TAC ATC CTG ACC CAC TT
Reverse	ATG CTC CTG CTT GAG TAT GT
β-actin	
Forward	GTG GGC CGC CCT AGG CAC CAG
Reverse	GGA GGA AGA GGA TGC GGC AGT

boiled for 5 min at 95 ºC. Total cell lysates (10 ug) were applied on SDS- 8% polyacrylamide gels and transferred to PVDF membrane. The membranes were probed with anti-rabbit iNOS (BD Transduction Laboratories™, BD Biosciences, USA), anti-mouse COX-2 (BD Transduction Laboratories™, BD Biosciences, USA) and anti-rabbit-β-actin (Cell signaling, USA) antibodies. The western blot was visualized using an enhanced chemiluminescence detection kit (Amersham Bio-science, England) according to the manufacturer's instruction.

Reverse transcriptase and polymerase chain reaction (RT-PCR) – RAW 264.7 cells were seeded (1×10^6 cells/mL) in 100 mm cell culture plates containing 10 mL of culture medium. Cells were treated with test samples together with 1 ug/mL LPS. The RNA was isolated with RNA isoplus™ (Takara Biotechnology, Japan) and each RNA extract (2 ug) was reverse transcribed into cDNA using superscript II reverse transcriptase (Gendepot, USA). PCR was performed in 20 uL of a solution containing Top-Taq™ PreMix (CoreBio System, Korea). The primer sequences are listed in Table 1. After initial denaturation, 25 amplification cycles were performed for iNOS, COX-2, and β-actin. PCR products were separated by electrophoresis on 1% agarose gels.

Statistical analysis – All results were presented as the mean value ± standard deviation (S.D.) from three independent experiments. Significant differences between the control and the experimental groups were assessed by the Student's t-test. Results were considered significant at $p < 0.01$.

Results and Discussion

Compound **1** was obtained as a yellow plate and the mass spectrum of compound **1** was in accordance with the formula $C_{28}H_{40}O$. Absorption bands at 1664 and 1587 cm^{-1} in the IR spectrum confirmed the presence of carbonyl and double bonds. The ^{1}H-NMR spectrum showed the presence of angular methyl peaks at δ 0.89 and 0.92. Four doublets were present at δ 0.76, 0.77, 0.86, and 0.99 for the secondary methyl group. The presence of a double bond was further indicated by its signals at δ 5.13 (1H, d,d, J = 8.2 Hz, 15.3 Hz) and 5.19 (1H, d,d, J = 7.5 Hz, 15.3 Hz). Olefinic proton signals were presented at δ 5.66, 5.95, and 6.54. A total of 28 carbon signals were observed in the ^{13}C-NMR spectrum, including an methyl carbon signal at δ 16.61, 17.61, 18.91, 19.63, 19.96, and 21.19, olefinic protonated carbon at δ 122.93, 124.41, and 134.00, quaternary carbon at δ 156.17, 156.08, and 164.39, exo-double bonded carbon at δ 132.47 and 134.96, ketonal carbon at δ 132.47 and 134.96. Accordingly, the structure of compound **1** was elucidated as ergosta-4,6,8(14),22-tetraen-3-one (ETO). All assignments in the ^{1}H-NMR and ^{13}C-NMR were based on measurements of HSQC, HMBC and ^{1}H-^{1}H COSY. The final structure of compound **1** was confirmed by a comparison of spectroscopic values to those reported in the literature (Yuan *et al*., 2003; Gao *et al*., 2003; Gao *et al*., 2002; Liu *et al*., 2005; Lee *et al*., 2005) (Fig. 1).

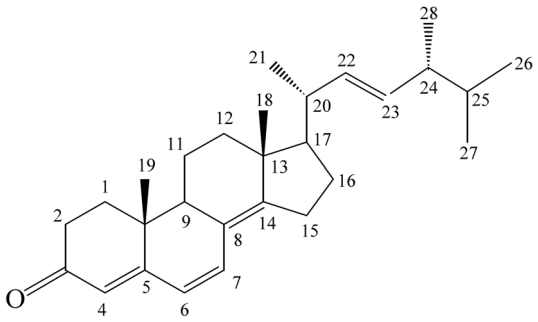

Fig. 1. Structure of ergosta-4,6,8(14),22-tetraen-3-one from *Phellinus pini*.

Compound **2** was purified as a colorless needle, and the molecular ion peak was found at *m/z* 398 in the EI-MS spectrum, suggesting that the molecular formula of compound **2** was $C_{28}H_{46}O$. The IR spectrum showed an absorption band at 3393 cm^{-1} due to hydroxyl group and 1715 cm^{-1} of double bond. The ^{1}H-NMR spectrum showed signals for two angular methyl protons at δ 0.54 and 0.79, and three secondary methyl protons at δ 0.85, 1.02, and 1.03 with J value of 7.0 Hz. Olefinic proton signals were observed at δ 5.18, 4.66, and 4.71. Terminal methylene signals were presented at δ 4.65 and 4.71. A total of 28 carbon signals were observed in the ^{13}C-NMR spectrum, including an methyl carbon signal at δ 22.06, 22.19, 19.04, 12.05, and 13.23, hydroxylated carbon at δ 71.29, olefinic protonated carbon at δ 117.68 and δ 139.74, exo-double bonded carbon at δ 157.02 and δ 106.17. These results suggested that compound **2** was an ergosterol derivatives. With all measurements of HSQC, HMBC and

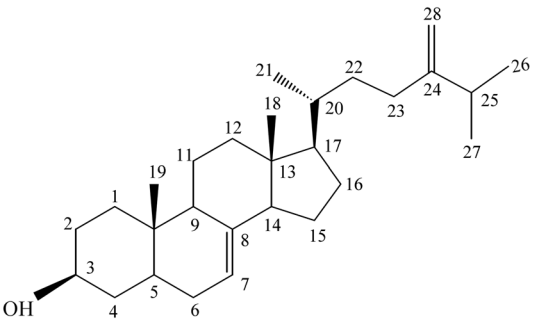

Fig. 2. Structure of ergosta-7,24(28)-dien-3-ol from *Phellinus pini.*

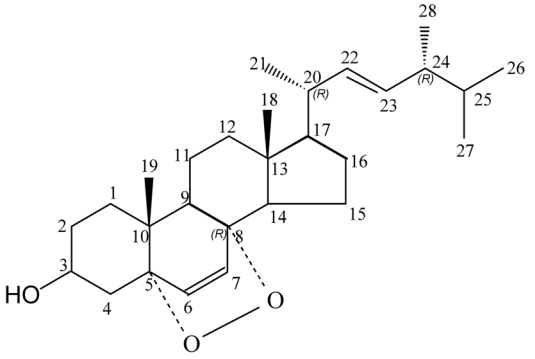

Fig. 3. Structure of 5,8-epidioxyergosta-6,22-dien-3-ol from *Phellinus pini.*

^{1}H-^{1}H COSY, and on a comparison of our data with published spectral data (Morris *et al*., 1974), the final structure of compound **2** was confirmed ergosta-7,24(28)-dien-3-ol (EDO) (Fig. 2).

Compound **3** was obtained as white needles and the mass spectrum of compound **3** was in accordance with the formula $C_{28}H_{44}O_3$. Absorption band at 3394 cm^{-1} in the IR spectrum confirmed the presence of hydroxyl group. The ^{1}H-NMR spectrum showed the presence of angular methyl peaks at δ 0.81 and 0.88. Four doublets were present at δ 0.82, 0.84, 0.90, and 0.99 for the secondary methyl group. The presence of olefinic protons was indicated by its signals at δ 6.23 and 6.48 with *J* value of 8.5 Hz. Double of doublets were presented at δ 5.15 (1H, 23-H, *J* = 8.4 Hz, 15.3 Hz) and 5.23 (1H, 22-H, *J* = 7.5Hz, 15.3Hz) for the double bond of side chain. A total of 28 carbon signals were observed in the ^{13}C-NMR spectrum, including an methyl carbon signal at δ 13.06, 17.75, 18.35, 19.82, 20.13, and 20.82, olefinic protonated carbon at δ 130.90 and 135.63, double bonded carbon at δ 135.39 and 132.49. Based on this observation, and on a comparison of our data with published spectral data (Sheffer *et al*., 1986; Takaishi *et al*., 1992; Sgarbi *et al*., 1997), compound **3** was identified as 5,8-Epidioxy-ergosta-6,22-dien-3-ol (EPO) (Fig. 3).

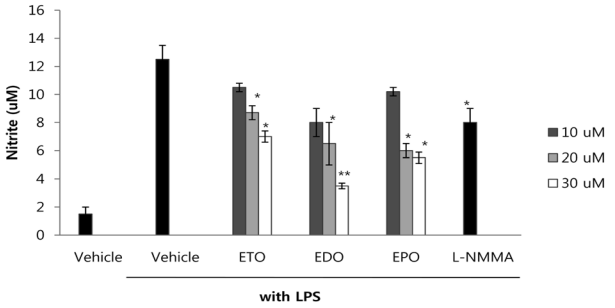

Fig. 4. Inhibition of NO production in RAW 264.7 cells by ETO, EDO, and EPO. Macrophage cells were treated with LPS (1 ug/mL) only or cotreated with L-NMMA (5 ug/mL) and ETO, EDO, and EPO (10 uM, 20 uM, and 30 uM) for 18 h. NO released into the cell culture medium was measured in the nitrite form by the Griess reagent. Each bar shows the mean ± S.D. of three experiments. *Indicates a significant difference from control, *$p < 0.01$, **$p < 0.001$.

NO is a low molecule radical that mediates neuro-transmission, blood coagulation, blood pressure, and immune-modulating processes (Stamlar *et al*., 1992), including diseases such as atherosclerosis, arthritis, inflammatory bowel disease, and other types of tissue injury (Bogdan *et al*., 2000; Raghav *et al*., 2007). NO synthesized from L-arginine by nitric oxide synthase at many cells and tissues (Galla, 1993). Inducible NO synthase (iNOS) is responsible for overproduction of NO in activated macrophages during inflammation. iNOS is over-expressed and induced in response to various immune modulating molecules such as lipopolysaccharide (LPS), interleukin (IL)-1, interferon gamma (IFN-r), and pro-inflammatory cytokines (Moncada *et al*., 1991; Li and Verma, 2002; Liu and Malik, 2006). Prostaglandin (PG) is another important mediator of inflammation and synthesized by cyclooxygenase (COX) (Maier *et al*., 1990; Lee *et al*., 1992). Overproduction of NO further enhances COX-2 activity, suggesting the cross talk between iNOS and COX-2 enzymes (Davis *et al*., 2001). Therefore, inhibition of NO production and expression of iNOS and COX-2 could provide targets for prevention and treatment of inflammatory diseases. To investigate the inhibitory activities on NO production, protein and m-RNA expression of iNOS and COX-2, three isolated compounds from the fruit body of *Phellinus pini* were treated in LPS-stimulated RAW 264.7 cells.

Our study demonstrated the anti-inflammatory effects of three ergosterol compounds on LPS-activated RAW 264.7 cells. Compounds **1**, **2**, and **3** inhibited NO production in RAW 264.7 cells at 10 uM, 20 uM, and 30 uM respectively. The inhibition was dose-dependent and showed significant inhibition at doses of 20 uM and 30

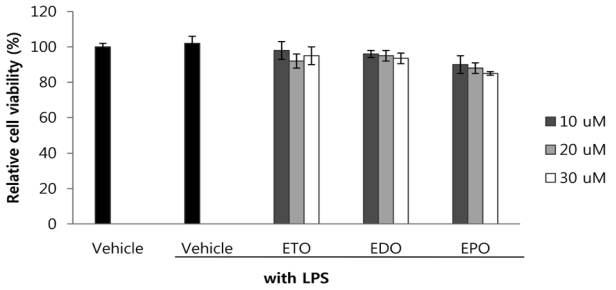

Fig. 5. Effects of ETO, EDO, and EPO on the viability of RAW 264.7 cells with LPS (1 ug/mL). The viability of the cells treated with LPS was measured by MTT assay. Results are expressed as % of negative control. Results are expressed as a % of the negative control. Each bar shows the mean ± S.D. of three experiments.

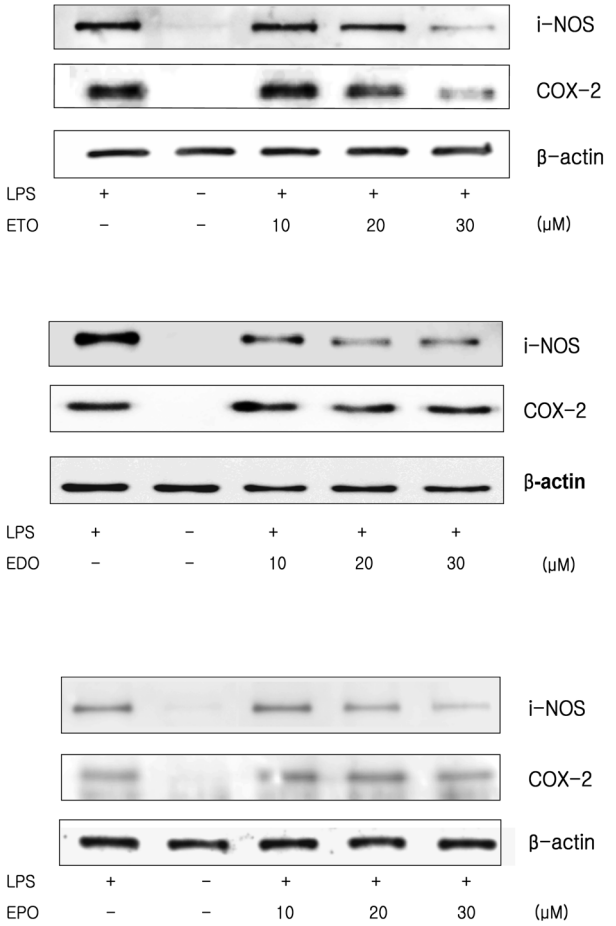

Fig. 6. Effects of ETO, EDO, and EPO on the expression of iNOS and COX-2 protein in LPS-activated RAW 264.7 cells. Cells were treated for 20 h with ETO, EDO, or EPO (10, 20, 30 uM) during LPS (1 ug/mL) activation. Cell lysates were prepared and the protein levels of iNOS, COX-2, and β-actin were determined by western blotting.

uM (Fig. 4). Compounds **1**, **2**, and **3** reduced NO production in the assay with IC_{50} values of 29.7 μM (**1**), 15.1 μM (**2**), and 18.4 μM (**3**) respectively; for L-NMMA,

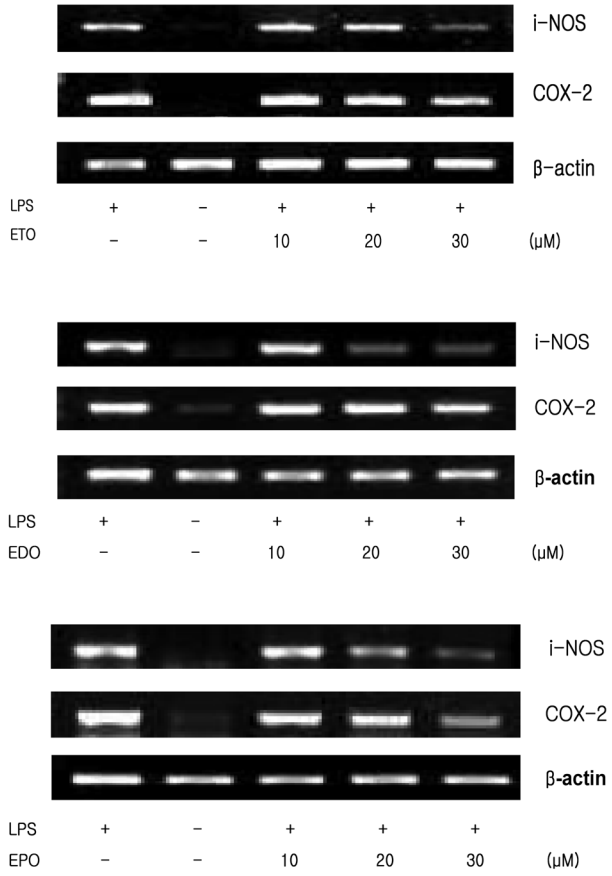

Fig. 7. Effects of ETO, EDO, and EPO on the expression of mRNA in LPS-activated RAW 264.7 cells. Cells were treated for 20 h with ETO, EDO, or EPO (10, 20, 30 uM) during LPS (1 ug/mL) activation. The mRNA levels of iNOS, COX-2, and β-actin were determined by RT-PCR from total RNA extracts.

a positive control, was 15.7 uM. This inhibition of NO production was not due to cytotoxicity, as determined by MTT assay. Treatment with these compounds and LPS inhibited NO production in concentration dependently without influence to cell viability (Fig. 5). To investigate the effects of compounds on iNOS and COX-2 protein expression, western blot analysis was performed to clarify the mechanism for the inhibition of NO production by compounds **1, 2,** and **3** in LPS-activated RAW 264.7 cells. The treatment of compound 1, 2, and 3 (10 uM, 20 uM, 30 uM) decreased the expression of iNOS protein level of RAW 264.7 cells dose dependently, but effect on expression of COX-2 protein level was minor than that of iNOS (Fig. 6). Next, the effects of compound 1, 2, and 3 on the expression of iNOS and COX-2 mRNA were analyzed by RT-PCR. As shown in Fig. 7, compounds 1, 2, and 3 showed a excellent suppression of mRNA expression at dose of 30 uM.

In conclusion, three ergosterol derivatives from *Phellinus*

pini inhibited LPS-induced iNOS and COX-2 expression and NO production with IC_{50} values of 29.7 μM (**1**), 15.1 μM (**2**), and 18.4 μM (**3**) in macrophage. Therefore, these results suggest that these compounds exert anti-inflammatory action via inhibition of iNOS and COX-2.

Acknowledgements

This Research was supported by the Sookmyung Women's University Research Grants 2011.

References

Ayer, W.A., Muir, D.J., and Chakravarty, P., Phenolic and other metabolites of *Phellinus pini*, a fungus pathogenic to pine. *Phytochemistry* **42**, 1321-1324 (1996).

Bogdan, C., Röllinghoff, M., and Diefenbach, A., The role of nitric oxide in innate immunity. *Immunol. Rev.* **173**, 17-26 (2000).

Davis, K.L., Martin, E., Turko, I.V., and Murad, F., Novel effects of nitric oxide. *Annu. Rev. Pharmacol. Toxicol,* **41**, 203-236 (2001).

Epimenko, O.M. and Ageenkova, L.V., Chemical composition of the wood-rotting fungus *Phellinus pini* var abietis. *Kormovye Belki Fiziol. Akt Veshchestva Zhivotnovod,* 107-109 (1965).

Galla, H.J., Nitric oxide, NO, an intracellular messenger. *Angew. Chem. Int. Ed. Engl.* **32**, 378-380 (1993).

Gao, J.M., Shen, J., Zhang, A.L., Zhu, W., Zhang, X., and Liu, J.K., Chemical constituents of the fungus *Leccinum extremiorientale. Youji Huaxue,* **23**, 853-857 (2003).

Gao, J.M., Zhang, A.L., Wang, C.Y., and Liu, J.K., Constituents from Basidiomycetes *Paxillus panuoides*. *Xibei Zhiwu Xuebao,* **22**, 391-395 (2002).

Ikegawa, T., Nakanishi, M., Uehara, N., Chihara, G., and Fukuoka. F., Antitumor action of some Basidiomycetes, especially *Phellinus linteus. Gann,* **59**, 155-157 (1968).

Jang, H.J. and Yang, K.S., Inhibition of nitric oxide production in RAW 264.7 macrophages by diterpenoids from *Phellinus pini. Arch. Pharm. Res.* **34**, 913-917 (2011).

Jeong, S.C., Cho, S.P., Yang, B.K., Jeong, Y.T., Ra, K.S., and Song, C.H., Immunomodulating activity of the exopolymer from submerged mycelial culture of *Phellinus pini. J. Microbiol. Biotechnol.* **14**, 15-21 (2004).

Lee, S.H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D., and Hwang, D., Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.* **267**, 25934-25938 (1992).

Lee, W.Y., Park, Y.K., Ahn, J.K., Park, S.Y., and Lee, H.J., Cytotoxic activity of ergosta-4,6,8(14),22-tetraen-3-one from the sclerotia of *Polyporus umbellatus*. *Bull. of the Kor. Chem. Soc*. **26**, 1464-1466 (2005).

Lee, S.M., Kim, S.M., Lee, Y.H., Kim, W.J., Park, J.K., Jang, W.J., Shin, H.D., Andriy, S., and Park, Y.I., Macromolecules isolated from *Phellinus pini* fruiting body: chemical characterization and antiviral activity. *Macromolecular Research* **18**, 602-609 (2010).

Li, Q. and Verma, I.M., NF-kappaB regulation in the immune system. *Nat. Rev. Immunol*. **2**, 725-734 (2002).

Liu, H.B., Cui, C.B., Gu, Q.Q., Cai, B., Zhao, Q.C., and Guan, H.S., Chemical constituents of *Pterocarya tonkinesis* and their antitumor activities in vitro. *Zhongguo Yaoxue Zazhi,* **40**, 414-416 (2005).

Liu, S.F. and Malik, A.B., NF-kappaB activation as a pathological mechanism of septic shock and inflammation. *Am. J. Physiol. Lung. Cell. Mol. Physiol*. **290**, L622-L645 (2006).

Maier, J.A., Hla, T., and Maciag, T., Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J. Biol. Chem.* **265**, 10805-10808 (1990)

Moncada, S., Palmer, R.M., and Higgs, E.A., Nitric oxide: physiology, Pathophysiology, and pharmacology. *Pharmacol. Rev.* **43**, 109-142 (1991).

Morris, D.C., Safe, S., and Subden, R.E., Detection of the ergosterol and episterol isomers lichesterol and fecosterol in nystatin-resistant mutants of *Neurospora crassa. Biochem. Genet.* **12**, 459-466 (1974).

Mosmann, T., Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).

Raghav, S.K., Gupta, B., Shrivastava, A., and Das, H.R., Inhibition of lipopolysaccharide-inducible nitric oxide synthase and IL-1β through suppression of NF-κB activation by 3-(1',1'-dimethyl-allyl)-6-hydroxy-7-methoxy-coumarin isolated from *Ruta graveolens* L. *Eur. J. Pharmacol.* **560**, 69-80 (2007).

Sgarbi, D.B., Silva, A.J., Carlos, I.Z., Silva, C.L., Angluster, J., and Alviano, C.S., Isolation of ergosterol peroxide and its reversion to ergosterol in the pathogenic fungus *Sporothrix schenckii. Mycopathologia* **139**, 9-14 (1997).

Sheffer, M., Fried, A., Gottlieb, H.E., Tietz, A., and Avron, M., Lipid composition of the plasma-membrane of the halotolerant alga, *Dunaliella salina. Biochim. Biophys. Acta* **857**, 165-172 (1986).

Stamlar, J.S., Singel, D.J., and Loscalzo, J., Biochemistry of nitric oxide and its redox-activated forms. *Science* **258**, 1898-1902 (1992).

Takaishi, Y., Adachi, R., Murakami, Y., Ohashi, T., Nakano, K., and Tomimatsu, T., A polyoxygenated steroid from *Lasiosphaera nipponica, Phytochemistry,* **31**, 243-246 (1992).

Yang, B.K., Park, J.B., and Song, C.H., Hypolipidemic effect of exo-polymer produced in submerged mycelia culture of five different mushroom. *J. Microbiol. Biotechnol.* **12**, 957-961 (2002).

Yuan, A., Yamamoto, K., Bi, K., Zhang, P., Liu, P., and Kano, Y., Studies on the marker compound for standardization of traditional Chinese medicine *Polyporus sclerotium. Yakugaku Zasshi* **123**, 53-62 (2003).

Received April 20, 2012
Revised May 21, 2012
Accepted June 22, 2012