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Article

The Preventive Potential of the Mycelial Submerged Culture of *Ophiocordyceps sinensis* against Neurodegenerative Diseases and Its Possible Active Compounds

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Abstract: The pharmacological effects of the fruiting body of *Ophiocordyceps sinensis* (Os) exerted antioxidant, anti-virus, and immunomodulatory, whereas the anti-inflammatory effects and active components of Os mycelial submerged culture (OMSC) are not known. This study aimed to investigate the active compounds in the fermented liquid (FLOS), hot water (WEOS), and 50-95% (EEOS-50, EEOS-95) ethanol extracts of OMSC and their anti-inflammatory effects and potential mechanisms in LPS-stimulated microglial BV2 cells. The results demonstrated that all of the OMSC extracts could inhibit NO production in BV2 cells. EEOS-95 exhibited the strongest inhibitory effects (41 % inhibitory ability at 500 µg/mL), and its ergosterol, GABA, total phenolic, and total flavonoid contents were significantly higher than those of the other extracts (18.60, 18.60, 2.28, and 2.14 mg/g, $p < 0.05$, respectively). EEOS-95 also has a strong inhibitory ability against IL-6, IL-1 β and TNF- α with IC₅₀ of 617, 277 and 507 µg/mL, respectively, which is higher than that of 1 mM melatonin. The anti-inflammatory mechanism of EEOS-95 seems to be associated with up-regulation of PPAR- γ /Nrf-2/HO-1 antioxidant-related expression and down-regulation of NF- κ B/COX-2/iNOS pro-inflammatory expression signaling. Therefore, we hypothesized that EEOS-95 can prevent neurodegenerative diseases, but the effect on the application *in vivo* should be further investigated.

Keywords: *Ophiocordyceps sinensis*; neurodegenerative disease; microglial; anti-inflammatory; cytokines; submerged culture

1. Introduction

The caterpillar fungus *Ophiocordyceps sinensis* (Os), known as Yarshagumba in Nepal and Dong-chong-xia-cai in Chinese, is found in the alpine regions of Bhutan, India, China, and Nepal [1,2]. *O. sinensis* has been treasured for centuries in traditional medicine. Primarily used as a tonic to boost immunity, it holds immense potential for various therapeutic applications. Several studies have documented its diverse beneficial properties, making it a fascinating subject for scientific exploration. Previous research has highlighted the potential of Os in addressing a multitude of health concerns. Some of its notable attributes in nervous system diseases, immunomodulatory, renal, liver, cardiovascular; especially, it inhibited the severe acute respiratory syndrome virus (SARS virus) 2003 in China [3,4]. Besides, Os exerted an excellent source of phytochemicals such as phenolic acids, amino acids, fatty acids, sterols, polysaccharides, nucleosides, etc... which have contributed to various beneficial nutritional or pharmacological properties [4]. Os traditionally consumed in powdered form, mixed with milk or water, or added to tea or soup. The high price of Os is mainly

due to its medicinal value, which has led to an increasing demand from consumers and a gradual decrease in the production of natural wild species (fruiting bodies). Wei et al. warn that climate change further threatens its wild distribution [5]. Based on the above reasons, some researchers have carried out mycelium isolation technology and used the isolated *Os* mycelial for submerged culture [3]. The benefits of this new approach are twofold: it reduces pressure on wild populations and promotes ecological sustainability, while providing a more economically viable source of *Os*. Although many studies have been conducted in the past to demonstrate the effects of fruiting body of *Os* on human health, the functionality of submerged cultures of *Os* mycelia and the active compounds in mycelia and fermentation still need to be further verified.

Neurodegenerative diseases (NDs) are a group of progressive conditions that affect millions of people worldwide, predominantly in their later years. These devastating disorders attack the nervous system, particularly the brain, leading to a decline in cognitive and motor function. Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) are just a few examples of the many faces of NDs. The rising number of ND cases puts immense pressure on healthcare systems, families, and communities [6–10]. While the specific causes of each NDs vary, researchers have identified several key factors that contribute to their development, these include oxidative stress (an imbalance between free radicals and antioxidants) leading to cellular damage, protein aggregation (the abnormal buildup of misfolded proteins in the brain, forming toxic clumps), neuro-inflammation (chronic inflammation in the nervous system damaging brain cells), neurotransmission impairment (disruption of chemical communication between neurons), mitochondrial dysfunction (impaired energy production in brain cells), excitotoxicity (excessive stimulation of brain cells, leading to their death) [11].–

Neuroinflammation is a defensive response of the brain to injury or infection, occurring in both chronic and acute neurodegenerative disorders. Microglia, the innate immune cells of the central nervous system, become rapidly activated when the brain is infected, injured, or damaged. They regulate the production of proinflammatory cytokines and immune response mediators, including nitric oxide (NO), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), reactive oxygen species (ROS), and various neurotoxic mediators [12,13]. Therefore, inhibiting the activation of microglia and neuroinflammation is a potential therapeutic target for reducing neurodegenerative diseases and neuronal damage.

The important bioactive compounds detected in *Os* mycelia are as follows: adenosine, polysaccharides, ergosterol, cordycepin, etc. [14,15]. These compounds have been reported to have various biological and pharmacological effects such as immunomodulatory, anti-inflammatory, and antioxidant. [3]. Li et al. reported that bioactive compounds of mycelia isolated from wild *Os* had neuroprotective effects on PC12 cells and prevented hydrogen peroxide-induced nerve damage in rats [16]. However, to date, no analytical data are available on the effects of OMSC on BV2 microglia and the analysis of the bioactive components of their mycelial extracts.

Thus, in the present report, we explored the contents of active compounds of fermented liquid (FLOS), and various mycelial extracts (hot water -WEOS, 50 % -EEOS-50, and 95 % -EEOS-95 ethanol extracts) of *Os* mycelial submerged culture (OMSC) by RP-HPLC assayed and evaluated their potential on cytotoxicity and mediator NO production. Then, their anti-inflammatory effects and possible mechanisms of OMSC in microglial BV2 cells induced by lipopolysaccharides (LPS) will be studied.

2. Materials and Methods

2.1. Chemicals

The mouse microglial BV2 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C). Lipopolysaccharide (LPS; Escherichia coli O26:B6) and bovine serum albumin were bought from Sigma-Aldrich Co. (St Louis, MO, USA). RPMI 1640, and fetal bovine serum (FBS) from Gibco by Life Technologies (Frederick, MD, USA). Chemicals and

reagents are of high quality. Tips, dishes, test tubes, etc. for cell culture were bought from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Preparation of Hot Water and Ethanol Extracts from *Ophiocordyceps Sinensis* Submerged Culture

Ophiocordyceps sinensis (BCRC 930166) were collected from wild *Ophiocordyceps sinensis* (Qinghai Province, China), and identified by Bioresource Collection and Research Center (Hsinchu, Taiwan, R.O.C.). This experiment used the dried mycelia (Os H101) kindly provided by Professor Hsu Tai-Hao. Os H101 were cultured at pH 7.0, 100×g, 18 °C, and 15 days in a 2-L Erlenmeyer flask containing (3% glucose, 0.5% peptone, 0.3% yeast extract, 0.1% H₂PO₄, and 0.05% MgSO₄·7 H₂O) on a rotary shaker (120 rpm). At the end of the fermentation process, the mycelial submerged culture of Os H101 (OMSC) was harvested. OMSC was extracted with a 1:20 ratio (w/v) of 121 ± 2 °C hot water for 15 mins; or extracted with a 1:20 ratio of 50%, 95% ethanol by soaking at room temperature for 24 h. The extracts were centrifuged (1500 rpm, 10 min) and concentrated at reduced pressure by using a rotary evaporator. Then, the OMSC extracts were lyophilized to obtain fermented liquid of Os (FLOS), 90°C hot water extract of Os (WEOS), 50 % ethanol extract of Os (EEOS-50), 95 % ethanol extract of Os (EEOS-95), respectively. Finally, samples were stored at 4°C for the following analysis. The extraction yield of WEOS (32.9%), EEOS-50 (25.1%), EEOS-95 (7.4%), respectively.

2.3. Determination of Bioactive Compounds

The bioactive compounds of OMSC were carried out using an Agilent 1200 reversed phase High-performance liquid chromatography coupled with a diode-array detector (Hitachi, Chiyoda City, Japan, Chromaster 5430). A HIQ Sil C18W reversed-phase column was used (4.6 mm × 250 mm, 5 µm). The results were expressed in mg/g. All solvents were of HPLC grade and filtered before HPLC analysis.

2.3.1. Measurement of Adenosine

Adenosine was determined by applying the method described in Chang et al., [17] with a slightly modified. The sample or standard was dissolved in 1 mL distilled water, and ultrasound-assisted (ultrasonic power 100 W) extracted for 1 h. The mobile phase consisted of methanol:0.02 M KH₂PO₄ (15:85, v/v). The flow rate was 0.7 mL/min, absorbance was measured at 254 nm, and the injection volume was 20 µL.

2.3.2. Measurement of Ergosterol

Ergosterol was detected as described by Yuan et al., [18]. Sample or standard (10 mg) was dissolved in 1 mL methanol/ dichloromethane (75: 25, v/v), and shaken for 1 h. The mobile phase consisted of solvents A (80% methanol) and B (75% methanol in dichloromethane) with gradient elution as follows: 0–5 min (100% A); 5–19 min (0%–100% B); 20 min (100% B); 34–35 min (100% B). The flow rate was 1.0 mL/min, absorbance was measured at 280 nm, and the injection volume was 20 µL.

2.3.3. Measurement of GABA

Measurement of GABA was performed as previously described by Rogério da Silva Moraes et al., [19]. The mobile phase was A: composed of 50 mM sodium acetate, 5% methanol, and 2-propanol (pH 5.67) and B: 70% methanol. The gradient is as follows: 0–10 min: 100% A, 10–20 min: 70% A, 20–25 min: 50% A, 25 min: 100% A. The flow rate was 0.8 mL/min, absorbance was measured at 338 nm, and the injection volume was 20 µL.

Prepare chemical as follows: derivatization working solution (OPA-NAC complex) to allow reaction between OPA and NAC thiol group: 16.3 mg N-acetylcysteine (NAC), 13 mg o-phthalaldehyde (OPA), 300 µL methanol was react for 30 min, room temperature, dark, stored in plastic test tubes at 4°C, in dark (used within 24 hours after preparation); and borate buffer (pH 9.6).

GABA standard or samples were mixed: 60 μ L standard or sample, 40 μ L borate buffer, and 10 μ L OPA-NAC complex; after 10 min of reaction, vortex this final solution prior to HPLC analysis.

2.3.4. Measurement of TPC, TFC, and EPS Contents

Extracellular polysaccharides (EPS) were detected following the phenol-sulfuric colorimetric method described by Jiménez et al., [20].

Total phenolic content (TPC) and total flavonoid content (TFC) were measured by applying the Folin-ciocalteu and aluminum chloride colorimetric methods according to the method presented in Yang et al., [21].

2.4. Cytotoxicity Test of OMSC on BV2 Microglial

The mouse microglial BV2 cell line was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C). Cells were cultured in RPMI 1640, containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, and 1% antibiotic penicillin/streptomycin. The medium for cells used in the experiments was changed every 2 days, incubated at 37 °C and 5% CO₂.

The cytotoxicity evaluation of OMSC on BV2 microglial cells was determined by the MTT method described in Vo et al., [22]. Cells were treated with various concentrations (10–1000 μ g/mL) of FLOS, WEOS, EEOS-50, and EEOS-95; and cell viability was detected at 490 nm using an ELISA reader (Synergy HTX, BioTek, Winooski, VT, USA).

2.5. Measurement of Nitrite Production

The evaluation effect of OMSC on NO level was carried out by measuring nitrite accumulation as described in Vo et al. [22] with minor modifications. BV2 cells (5×10^5 cells/mL) were seeded in 24 well plates for 2 h. Cells were treated with 10–1000 μ g/mL of FLOS, WEOS, EEOS-50, and EEOS-95 for 24 h, respectively. LPS-stimulated (1 μ g/mL) for 24 h, and nitrite levels were detected at 540 nm using an ELISA reader (Synergy HTX, BioTek, Winooski, VT, USA).

2.6. Anti-Inflammatory Activities of 95% Ethanol Extract OMSC (EEOS-95)

2.6.1. Measurement of Pro-Inflammatory Cytokines Level

BV2 cells (5×10^5 cells/well) were seeded in 24 well plates for 2 h and treated with different concentrations (50–500 μ g/mL) EEOS-95 for 24 h, then incubated for 24 h with 1 μ g/mL LPS to induce the secretion of inflammatory-related cytokines. After incubation, the concentration of cytokines in the culture medium was determined using an ELISA kit according to the manufacturer's instructions. Cytokines were measured as IL-1 β , IL-6, TNF- α (Invitrogen Co. Camarillo, CA, USA), and PGE₂ (Life Technologies Corp. Frederick, MD, USA).

2.6.2. Western Blotting Analysis

Cell pellets were harvested and washed with cold phosphate buffer saline (pH 7.4). The cytoplasmic proteins 50 μ g were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to a poly-vinylidene fluoride membrane. The membrane was blotted and incubated with specific primary antibodies overnight at 4 °C, followed by incubation with horseradish peroxidase-conjugated secondary antibody. Finally, the blots were probed using enhanced chemiluminescence and autoradiographed. The relative density of protein expression was quantified using ImageJ software, developed by Wayne Rasband at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin, USA). The protein content in the supernatant was determined using the BCA protein assay kit (Thermo, Rockford, IL, USA). Western blot analysis detected the protein expression of iNOS, COX-2, NF- κ B, HO-1, Nrf-2, PARP- γ , and β -actin in the BV2 microglial cells.

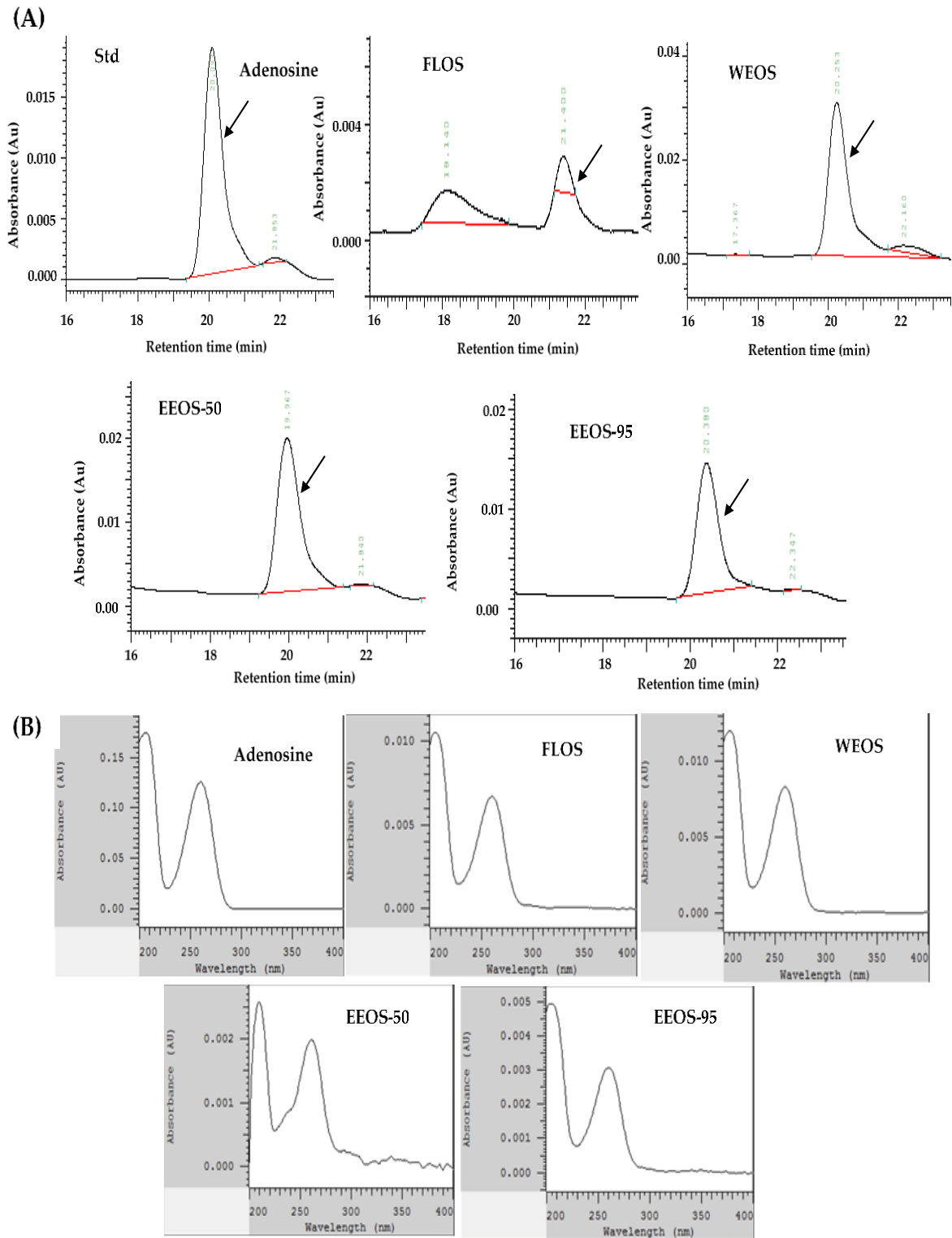
2.7. Statistical Analysis

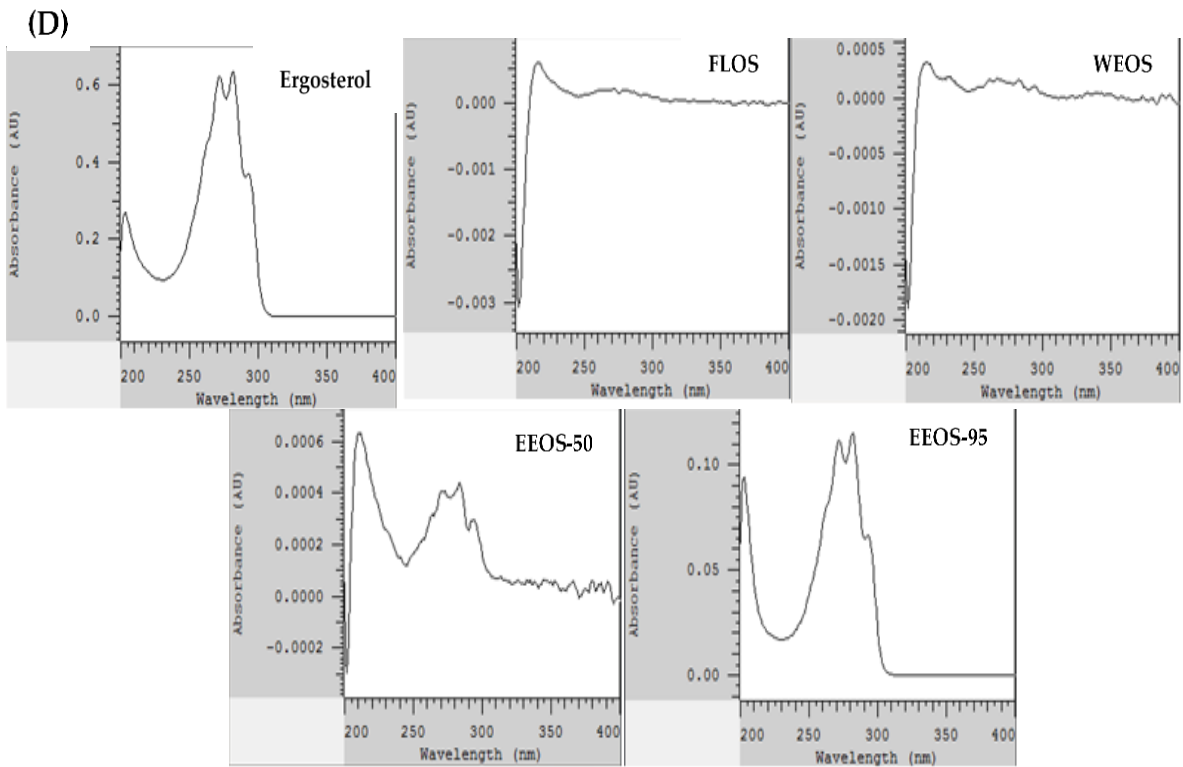
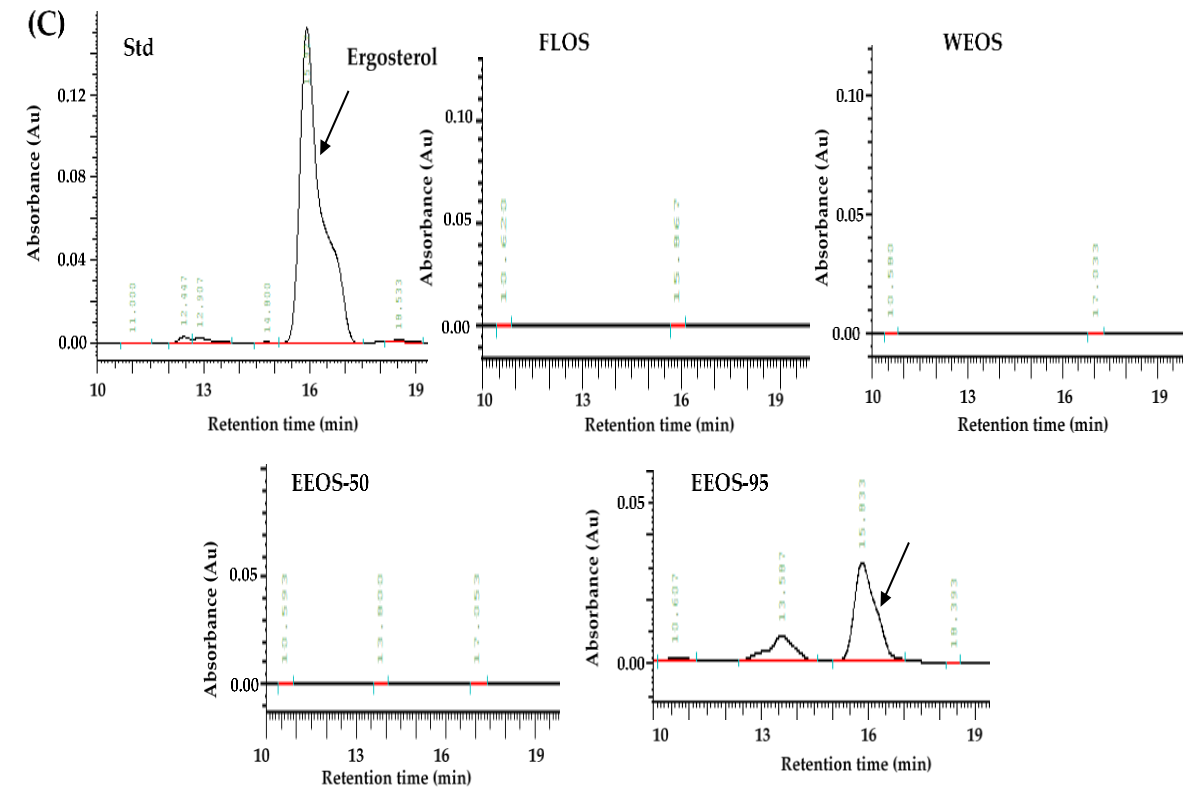
All statistical analyses were performed using SPSS for Windows, version 18 (SPSS Inc., Chicago, IL, USA). Data are expressed as means \pm standard deviation and analyzed using one-way ANOVA followed by Duncan's multiple range test. $p < 0.05$ is considered statistically significant.

3. Results

3.1. Bioactive Compounds in Extract of OMSC

As shown in Figure 1, the HPLC profile standard compounds of adenosine, ergosterol, and GABA; and various extracts of *Os mycelia* were detected by the HPLC-DAD-UV/Vis system. Figure 1 A, E, the chromatogram shows that adenosine and GABA were found in all extracts of OMSC following FLOS, WEOS, EEOS-50, and EEOS-95. The full wavelength of the sample is compared with that of the standard and has the same waveform. (Figure 1 B, F). However, the ergosterol peak was only detected in EEOS-95 (Figure 1 C), as evidenced by the ergosterol standard full-wavelength waveform. (Figure 1 D). As shown in Table 1, the dried matter of the fermented liquid of OMSC (FLOS) was rich in adenosine and polysaccharides (2.12 and 107.60 mg/g, respectively). Except for adenosine and polysaccharides (2.83 and 156.30 mg/g, respectively), GABA was also determined in the hot water extract of OMSC (WEOS) (12.60 mg/g), and the content was significantly lower than that of EEOS-95 (18.60 mg/g) ($p < 0.05$). It is noteworthy that EEOS-95 was significantly higher ($p < 0.05$) in all active compounds except adenosine and polysaccharides which were lower than the other extracts, especially ergosterol (18.60 mg/g), while its total polyphenols, total flavonoids, and GABA were 2.28, 2.14, and 18.6 mg/g, respectively.





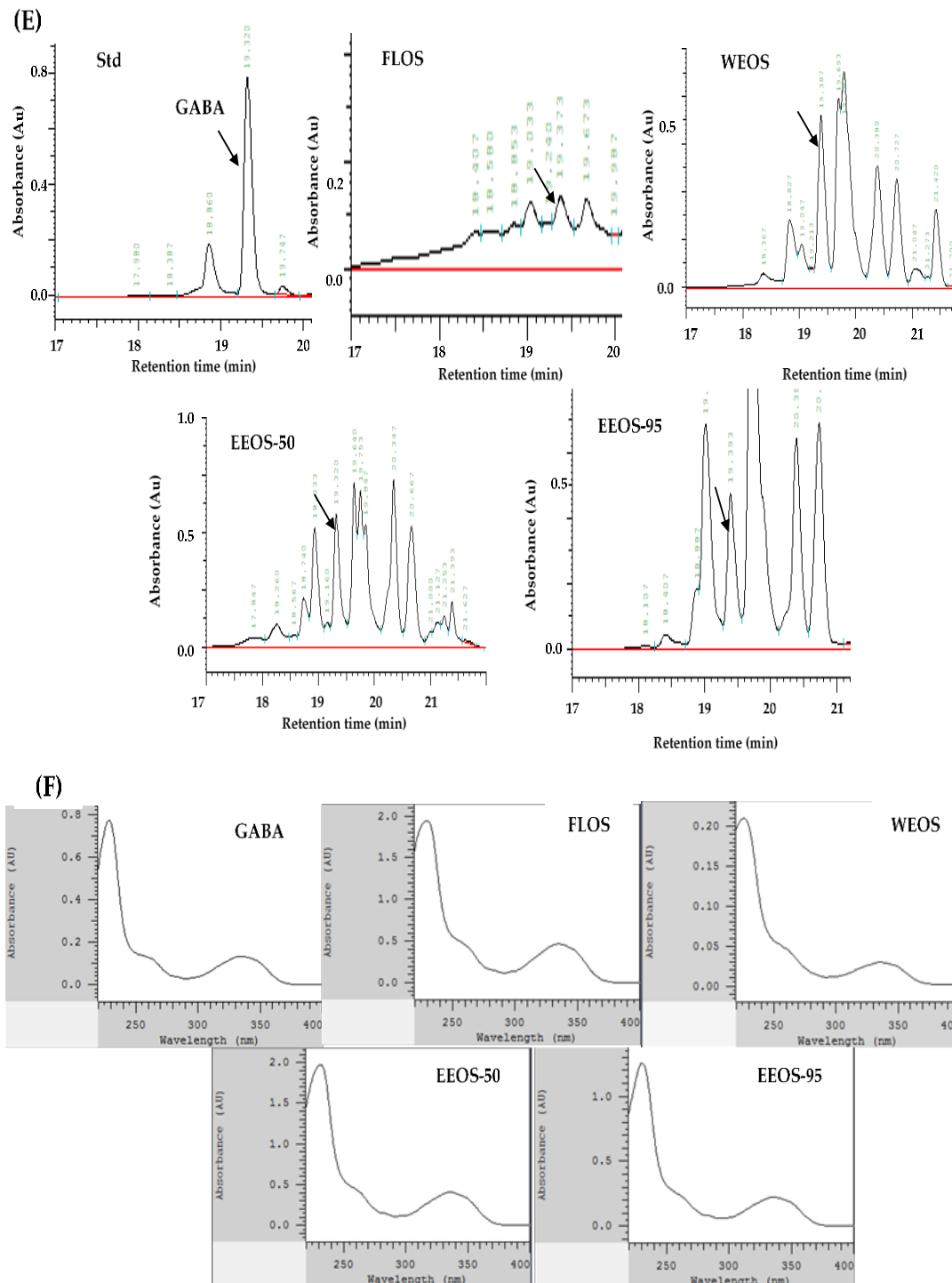


Figure 1. High-performance liquid chromatography chromatograms and wavelengths of Adenosine, Ergosterol, GABA compounds, and extracts of the mycelial submerged culture of *Os* H101 (OMSC). Chromatograms (A, C, and E) show standards of adenosine (A), ergosterol (C), GABA (E), and various extracts of OMSC, respectively. A diode array detector (DAD) was used to detect the full-wavelength (200–400 nm) comparison waveforms of standards and different extracts of OMSC (B, D, F). FLOS: fermented liquid of OMSC, WEOS: hot water extract of OMSC, EEOS-50: 50 % ethanol extract of OMSC, EEOS-95: 95 % ethanol extract of OMSC.

Table 1. Bioactive ingredients of various extracts from OMSC.

Compounds (mg/g)	Extracts ¹			
	FLOS	WEOS	EEOS-50	EEOS-95
Adenosine	2.12 ± 0.09 ^{b2}	2.83 ± 0.05 ^c	3.29 ± 0.29 ^d	0.19 ± 0.01 ^a
Ergosterol	ND	ND	ND	18.60 ± 0.70
Polysaccharide	107.60 ± 10.20 ^b	156.30 ± 7.10 ^c	28.50 ± 7.70 ^a	ND
Total Polyphenols	1.57 ± 0.09 ^a	1.71 ± 0.07 ^{bc}	1.77 ± 0.15 ^c	2.28 ± 0.05 ^d
Total Flavonoids	1.34 ± 0.01 ^b	1.17 ± 0.02 ^a	1.65 ± 0.01 ^c	2.14 ± 0.06 ^d
GABA	3.70 ± 0.30 ^a	12.60 ± 0.80 ^b	13.20 ± 0.60 ^b	18.60 ± 0.50 ^c

¹FLOS: fermented liquid of OMSC, WEOS: hot water extract of OMSC, EEOS-50: 50 % ethanol extract of OMSC, EEOS-95: 95 % ethanol extract of OMSC. ND: Non-detected. ²Values (means ± SD, n = 3 for the test groups) not sharing the same superscript letter in a row are significantly different (p < 0.05).

3.2. Cytotoxicity Evaluation of OMSC

The cytotoxicity of OMSC mycelia extracts and FLOS (50-1000 µg/mL) in BV2 microglial cells was evaluated using MTT analysis after 24 h incubation. Table 2 indicated that treatment of all extracts at concentrations up to 1000 (µg/mL) was not effective on cell survival of BV2 cells (p < 0.05) (cell viability > 90%). Thus, there was no toxic effect when BV2 cells were treated with 50 to 1000 (µg/mL) of OMSC extracts. The results also indicated that below a concentration of 500 µg/mL, the highest percentage of proliferation increased by EEOS-95 (about 68.5%). At a concentration of 1000 µg/mL, the cell viability of all the samples began to show a decreasing trend.

Table 2. Effect of various extracts of OMSC on cytotoxicity in BV2 microglial cells.

Treatments ¹	Cell viability (% of CON)			
	FLOS ²	WEOS	EEOS-50	EEOS-95
50	89.80 ± 13.20 ^{a3}	95.90 ± 7.20 ^a	94.30 ± 11.90 ^a	116.60 ± 8.60 ^a
100	106.40 ± 25.60 ^a	96.70 ± 1.40 ^a	102.20 ± 6.00 ^a	137.30 ± 12.60 ^b
250	119.40 ± 18.30 ^{ab}	108.80 ± 5.10 ^a	116.80 ± 6.20 ^{ab}	171.70 ± 12.90 ^{cd}
500	134.20 ± 11.30 ^b	123.70 ± 11.90 ^b	125.70 ± 4.40 ^b	196.50 ± 13.80 ^d
1000	114.00 ± 23.40 ^{ab}	116.50 ± 8.40 ^{ab}	123.00 ± 1.00 ^b	175.90 ± 3.00 ^c

¹Cells were pretreated with various extracts of OMSC (50-1000 µg/mL) for 24 h. The viability was measured by MTT assay. ²FLOS: fermented liquid of OMSC, WEOS: hot water extract of OMSC, EEOS-50: 50 % ethanol extract of OMSC, EEOS-95: 95 % ethanol extract of OMSC. ³Values (means ± SD, n = 3 for the test groups) not sharing the same superscript letter in a column are significantly different (p < 0.05).

3.3. Effect of *Os Mycelia* on Nitric Oxide

We examined the effect of various extracts of OMSC on LPS-induced NO production by Griess reagent assay (Table 3). BV2 cells were stimulated with LPS (1 µg/mL), and the NO level significantly increased (0.78 nmol/10⁶ cells) compared with the control (0.33 nmol/10⁶ cells) (p < 0.05). Our results also indicated that cells pre-treated with different concentrations (50-500 µg/mL) of extracts from OMSC mycelia-WEOS, EEOS-50, and EEOS-95 significantly suppressed the NO production by LPS-stimulated BV2 cells in a dose-dependent manner (p < 0.05), the inhibition effects at 500 µg/mL were 25, 26 and 41%, and EEOS-95 had the highest inhibition effect on NO production. FLOS also inhibited 25% of NO levels; however, there was no significant difference among treatment doses. (p < 0.05).

Table 3. Effect of various extracts of OMSC on lipopolysaccharide-induced nitrite production in BV2 microglial.

Treatments ¹	Nitrite concentration (nmol/10 ⁶ cells)			
		50	250	500
CON		0.33 ± 0.05 ^{a4}	-	-
LPS (1 µg/mL)		0.78 ± 0.12 ^d	-	-
MT (1mM) ²		0.45 ± 0.03 ^b	-	-
FLOS ³	+ LPS	-	0.55 ± 0.00 ^c	0.59 ± 0.07 ^c
WEOS		-	0.75 ± 0.02 ^d	0.74 ± 0.02 ^d
EEOS-50		-	0.76 ± 0.11 ^d	0.66 ± 0.05 ^{cd}
EEOS-95		-	0.64 ± 0.16 ^{cd}	0.53 ± 0.09 ^c
			0.46 ± 0.02 ^b	

¹Cells were pretreated with 1 mM melatonin (MT) or various extracts of OMSC (µg/mL) for 24 h and then incubated with lipopolysaccharide (LPS, 1 µg/mL) for 24 h. ²Melatonin (MT, N-acetyl-5-methoxytryptamine) is an animal hormone that exhibits physiological functions such as improving sleep, delaying aging, etc... MT is a positive control. ³FLOS: fermented liquid of OMSC, WEOS: hot water extract of OMSC, EEOS-50: 50 % ethanol extract of OMSC, EEOS-95: 95 % ethanol extract of OMSC. ⁴Values (means ± SD, n = 3 for the test groups) not sharing the same superscript letter in a column are significantly different (p < 0.05).

3.4. Anti-Inflammation Effect of EEOS-95 on Lipopolysaccharide-Induced BV2 Microglial Cells

We further tested EEOS-95, which affected the cytokine production induced by LPS in BV2 microglial cells. The level of IL-1β, TNF-α, IL-6, and PGE₂ was determined by ELISA kit. As shown in Table 4, LPS markedly increased IL-1β, IL-6, TNF-α, and PGE₂ levels, as compared with untreated controls (p < 0.05); however, all of these pro-inflammatory cytokines were significantly decreased by EEOS-95 (50-500 µg/mL) in a concentration-dependent manner. In addition, at a concentration of 500 µg/mL, the inhibitory effects of EEOS-95 on IL-1β, IL-6, TNF-α and PGE₂ production (96.60, 70.33, 49.83, and 43.57%, respectively, p < 0.05) were significantly better than that of 1 mM MT (30.22, 61.60, 11.40 and 6.70%, respectively, p < 0.05). The inhibition ratio of EEOS-95 on different cytokines showed that EEOS-95 had the best inhibition effect on PGE₂, followed by IL-1β, TNF-α, and IL-6, with inhibition rates of 90, 60, 50, and 40%, respectively, p < 0.05, at a concentration of 500 µg/mL (Figure 2).

Table 4. Effect of EEOS-95 on lipopolysaccharide-induced cytokines in BV2 microglial.

Treatments ¹	Cytokines			
	IL-1β (pg/mL)	TNF-α (ng/mL)	IL-6 (ng/mL)	PGE ₂ (pg/mL)
CON	4.29 ± 1.30 ^{a3}	13.71 ± 0.69 ^a	0.00 ± 0.00 ^a	97.60 ± 5.50 ^a
LPS (1 µg/mL)	76.66 ± 3.47 ^f	70.54 ± 4.89 ^d	608.22 ± 17.40 ^e	933.90 ± 12.30 ^e
MT (1 mM) ²	54.79 ± 0.94 ^d	35.53 ± 3.58 ^b	538.87 ± 28.22 ^{de}	877.90 ± 10.30 ^d
50	+ LPS	62.86 ± 3.90 ^e	49.58 ± 0.75 ^c	510.21 ± 7.09 ^{cd}
250		36.59 ± 1.54 ^c	53.10 ± 3.53 ^c	451.58 ± 2.73 ^c
500		25.76 ± 0.61 ^b	42.22 ± 3.22 ^{bc}	343.20 ± 1.86 ^b
				126.00 ± 27.90 ^a

¹Cells were pretreated with 1 mM melatonin or 50-500 (µg/mL) of EEOS-95: 95 % ethanol extracts of OMSC for 24 h and then incubated with lipopolysaccharide (LPS, 1 µg/mL) for 24 h. ²Melatonin (MT) is a positive control.

³Values (means \pm SD, n = 3 for the test groups) not sharing the same superscript letter in a column are significantly different ($p < 0.05$).

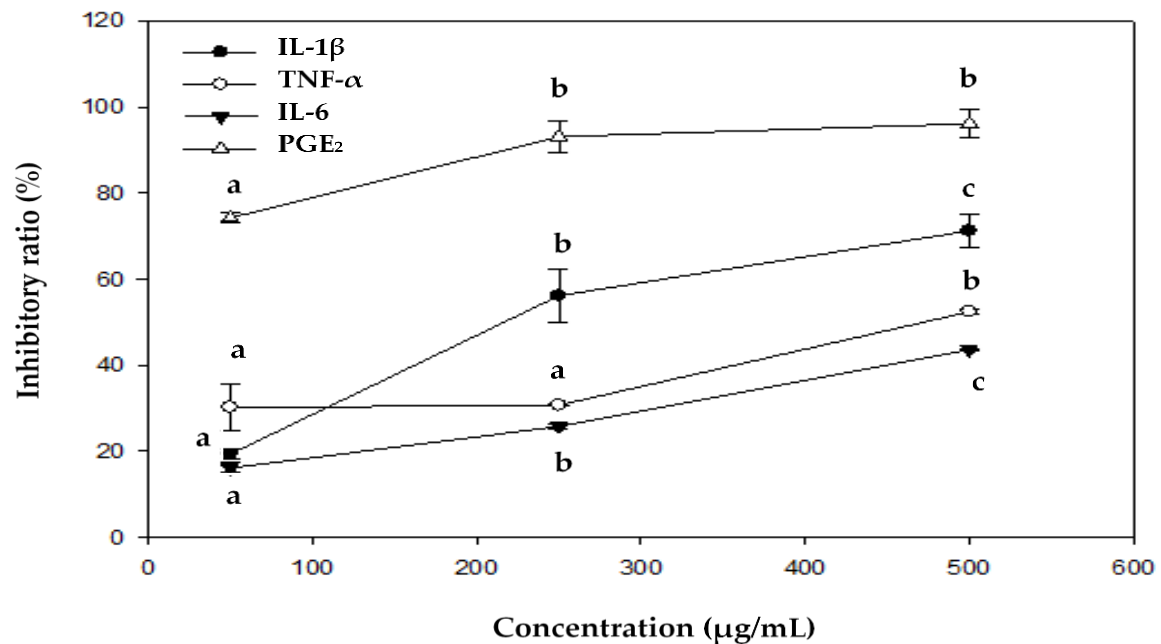


Figure 2. The inhibitory ratio of EEOS-95 on lipopolysaccharide-induced cytokines production in BV2 microglial cells. Values (means \pm SD, n = 3 for the test groups) not sharing the same superscript letter are significantly different ($p < 0.05$).

3.5. Effect of EEOS-95 on the Expression of Inflammatory Proteins in LPS-Induced BV2 Microglial

Western blot analyses were performed to determine whether EEOS-95 has a direct effect on the pro-inflammatory factor in LPS-induced BV2 microglial. As shown in Figure 3 (A), LPS significantly induced the expression of inflammation-associated proteins such as iNOS, COX-2, and NF- κ B, which were dose-dependently (50-500 μ g/mL) reduced by EEOS-95 ($p < 0.05$). At a concentration of 500 μ g/mL, EEOS-95 showed a 34, 55, and 20% decrease in protein expression of iNOS, COX-2, and NF- κ B, respectively, compared to the LPS-treated group alone (Figure 3B. C. D, $p < 0.05$). Pretreatment of BV2 cells with 1 mM melatonin also reduced the expression levels of 12% of iNOS and COX-2 and did not affect NF- κ B compared with the LPS group only.

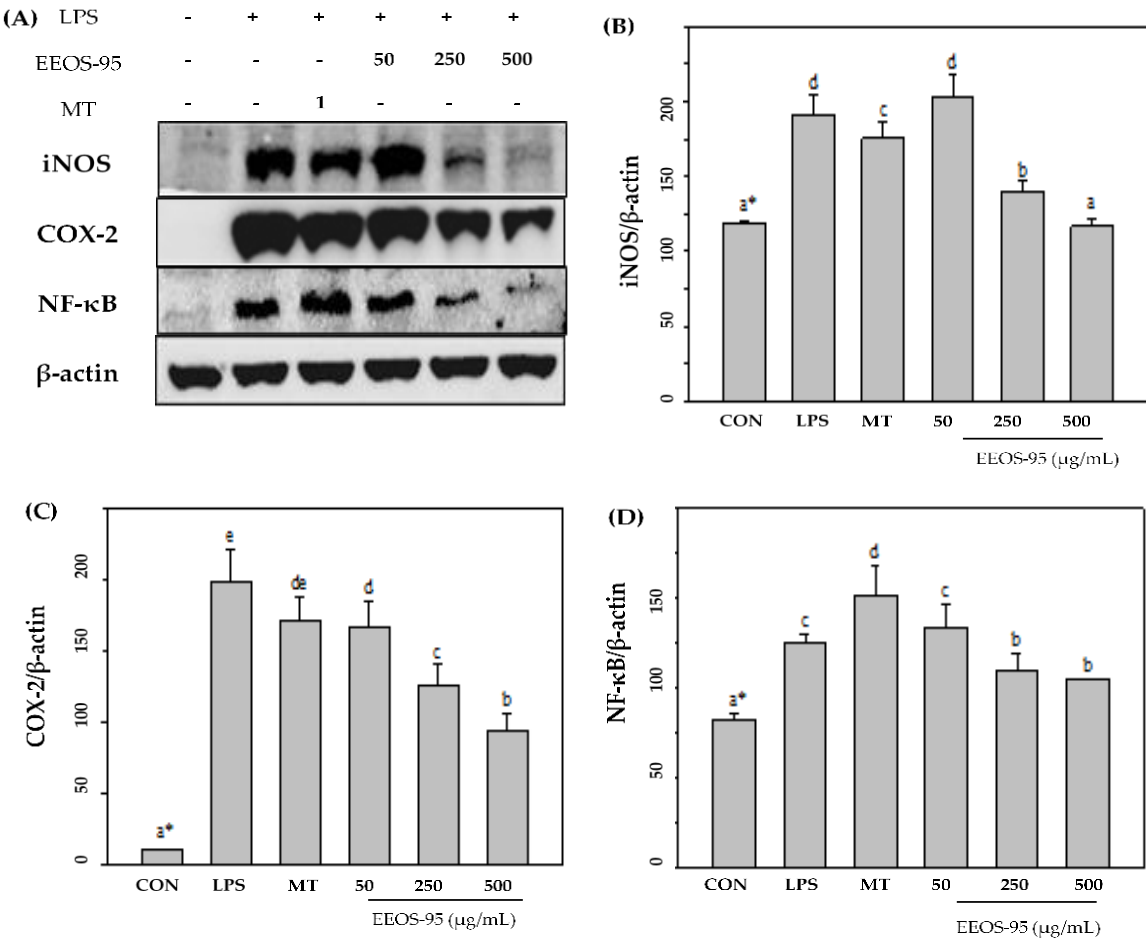


Figure 3. Effect of EEOS-95 on the expression levels of pro-inflammation factors in lipopolysaccharide-induced BV2 microglial. Cells were treated with EEOS95 (50-500 μg/mL) and MT (1 mM Melatonin) for 24 h and incubated with lipopolysaccharide (LPS, 1 μg/mL) for 24 h. (A) protein expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB) and β-actin were detected in the cytoplasm by Western blotting assay. The quantitative of (B) iNOS, (C) COX-2, and (D) NF-κB were analyzed by ImageJ software. Values (means ± SD, n = 3 for the test groups) not sharing the same superscript letter are significantly different (p < 0.05).

3.6. Effect of EEOS-95 on the Expression of Anti-Inflammatory Protein in LPS-Induced BV2 Microglial

As the results were shown in Figure 4, cells incubated with EEOS-95 significantly increased the expression levels of antioxidant factors (HO-1, Nrf-2, PPAR-γ) in the LPS-induced BV2 microglial. The protein expression level of HO-1, Nrf-2, and PPAR-γ in BV2 cells treated with EEOS-95 at 25-250 μg/mL showed a significant increase (p < 0.05) compared with the control group and the LPS-treated group. In contrast, EEOS-95 at a concentration of 500 μg/mL increased the expression of the control group by more than 50% and was superior to that of the 1 mM MT-treated group.

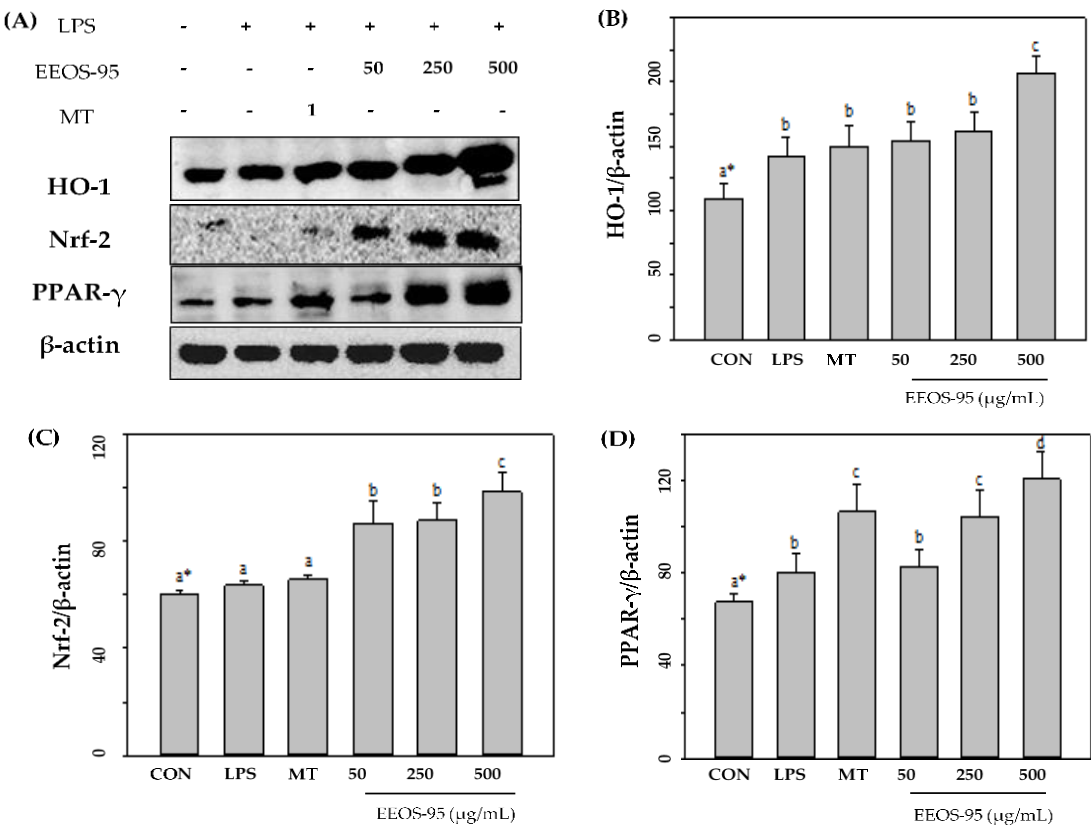


Figure 4. Effect of EEOS-95 on expression levels of antioxidant factors in lipopolysaccharide-induced BV2 microglial. Cells were treated with EEOS95 (50-500 μ g/mL) and MT (Melatonin 1 mM) for 24 h and incubated with lipopolysaccharide (1 μ g/mL) for 24 h. (A) protein expression of heme oxygenase-1 (HO-1), nuclear-related factor 2 (Nrf-2), peroxisome proliferator-activated receptors- γ (PPAR- γ), and β -actin were detected in the cytoplasm by Western blotting assay. The quantitative of (B) HO-1, (C) Nrf-2 and (D) PPAR- γ were analyzed by ImageJ software. Values (means \pm SD, n = 3 for the test groups) not sharing the same superscript letter are significantly different (p < 0.05).

4. Discussion

The wild caterpillar fungus *O*s is a traditional Chinese medicine, almost found in Asia for its rich bioactive profile and diverse therapeutic applications, also has over 30 bioactive compounds that exhibit numerous beneficial effects, including anti-inflammatory, antioxidant, anti-tumor, immunomodulatory, and anti-osteoporotic activities, proving effective in tackling ailments like diabetes, liver cancer, and kidney cancer [15,23,24]. Overharvesting of wild *O*s has significantly reduced its annual production, causing a gap between rising demand and declining supply. This has fueled research into sustainable alternatives, including artificial cultivation using *C. militaris* and submerged culture techniques for natural *O*s [24–28]. These approaches focus on optimizing culture conditions, maximizing mycelial biomass production, and exploring polysaccharide extraction. While substantial research has investigated the pharmacological potential of OMSC, a vital knowledge gap remains concerning the detailed analysis of their extracted bioactive components. Our study employed aqueous and ethanolic solvents to extract bioactive compounds from OMSC to identify their main bioactive components and anti-inflammatory potential.

Our quantitative analysis of the dried powder of OMSC extract using RP-HPLC revealed six bioactive ingredients: adenosine, ergosterol, polysaccharide, TPC, TFC, and GABA. Briefly, Adenosine belongs to nucleoside, which is an essential bioactive compound in the organism. The content of adenosine is 3.29 mg/g (EEOS-50), which was similar to previous reports (3.06 mg/g) and remarkably higher than that of natural *O*s [15]. Zhang et al. also pointed out that adenosine is

neuronal protection in NDs such as Alzheimer's and Parkinson's disease, suggesting its value as a marker for quality control in Os submerged fermentation [29]. However, the presence of these diverse bioactive compounds warrants further investigation into their potential health benefits and applications.

Ergosterol is an essential component of fungal cell membranes called main fungal sterol; its function is similar to cholesterol in animals, which can be converted to vitamin D2 under ultraviolet radiation or sunlight. The vegetarian body needs every day to provide vitamin D through a source of mushrooms; thus, ergosterol is interesting in further research [30]. The pharmacological effects of ergosterol have also been reported as an antioxidant, anti-inflammatory, anti-neurodegenerative, antimicrobial, anticancer, antidiabetic, etc... [31–39]. As reported by Peng and colleagues, ergosterol is the active compound of cultured mycelium *C. sinensis*, which has inhibited liver fibrosis [38]. Herein, we confirm that ergosterol is only present in EEOS-95 (18.60 mg/g), which is 6 times higher than other published data in the literature (3.20 mg/g) [14]. Thus, the potential of phytol-ergosterol from the OMSC will be interesting for future biomedical applications.

GABA is γ -aminobutyric acid, known as a non-protein amino acid, which has been biosynthesized from glutamic acid in the human body. GABA is considered the major inhibitory neurotransmitter in the central nervous system. GABA exhibited its inhibitory effect through various parts of the nervous system, including the cerebellum, hippocampus, hypothalamus, striatum, and spinal cord [40]. A previous study pointed out that biosynthetic GABA intake may have a beneficial effect on stress reduction and sleep improvement [41]. Surprisingly, the content of GABA in EEOS-95 (18.60 mg/g) is much higher than those of *C. militaris* (0.0686–0.180 mg/g) and *C. sinensis* (0.220 mg/g) [24,42]. Thus, the GABA-rich OMSC will be a potential source of natural neuroprotection.

Previous studies have addressed *Cordyceps* (*Ophiocordyceps*) *sinensis*, which contain polysaccharide compositions including arabinose, xylose, galacturonic acid, glucose, mannose, galactose, rhamnose [43,44]. These polysaccharides have exerted different pharmacological effects, involving antioxidant, anti-inflammatory, immunomodulatory, anti-cancer, and neuroprotection [15]. Nguyen et al. demonstrated that polysaccharides extracted from Os mycelia possess strong antioxidant effects and against hepatotoxicity in rats [45]. Additionally, three kinds of polysaccharides- glucose, mannose, and galactose isolated from Os, have strong antioxidation activity and neuroprotective properties against the free radical-induced neuronal cell toxicity on the cultured rat PC12 cells [16]. The content of polysaccharides in WEOS is 156.30 mg/g, which is similar to artificial cordyceps mycelia polysaccharide of submerged fermentation by previous reports (162.30 mg/g) [14]. Given their numerous potential health benefits, polysaccharides from cordyceps mycelia of submerged fermentation continue to be the focus of intense research and investigation in natural products with medicinal chemistry.

Evidence indicates polysaccharides can promote cell proliferation, scavenge cellular ROS, and downregulate the secretion of pro-inflammatory cytokines [46,47]. In the cell viability test, we also found that FLOS, WEOS, and EEOS-50 EEOS-95 promoted cell proliferation, which was presumably related to the polysaccharides contained in the samples. However, polysaccharides were not detected in EEOS-95, but their cell viability was higher than that of other extracts, suggesting that EEOS-95 may contain other cell proliferation components. Ergosterol is regarded as a "fungal hormone" that can stimulate growth and proliferation. Many studies have shown that ergosterol is essential for mitochondrial DNA maintenance in fungi, as cholesterol does in humans [48–50]. Ergosterol also exerts a differential effect on Androgen-dependent LNCaP and Androgen-independent DU-145 cancer cells; that is, ergosterol showed an antiproliferative effect on LNCaP and proliferative effect on DU-145. Thus, the promoted cell proliferative effect of EEOS-95 in BV2 cells (Androgen-independent cells) should contribute from the ergosterol.

Natural phenolic or flavonoid compounds, a class of bioactive compounds found in medicinal plants and mushrooms, are known for their antioxidant properties. These properties are directly attributed to their scavenging ability, provided by the hydroxyl groups within their structures, and contribute to improvements in human health. Our study demonstrated that flavonoids were extracted from medicinal plants (ex., daidzein, genistein, genistin, cajanol, and biochanin A), which

exhibit several pharmacological properties on antioxidant, oxidative stress anti-inflammation, anti-glycemic, and apoptosis [22,51]. TPC and TFC contents of fermented liquid (FLOS) and all mycelia extracts of *Os* submerged culture were 1.57~2.28 mg/g (TPC) and 1.17~2.14 mg/g (TFC). Tran et al [52] pointed out that the amounts of gallic acid, quercetin, quercitrin, and hesperidin dominated the *Ophiocordyceps Sobolifera* extracts at 193.60 142.07; 544.53 and 110.08 µg/g, respectively, out of a total of 990.27 µg/g dry weight active phenolic fraction, and these phenolic compounds of *Ophiocordyceps Sobolifera* extract were responsible for renal injury prevention [52]. Thus, we suggested that TPC and TFC present in OMSC extracts enhance the benefit of pharmacological properties of OMSC for functional foods. Our results revealed that EEOS-95 was the most potent extract in OMSC by inhibiting NO production induced by LPS in BV2 cells, which is related to their high contents of active compounds (ergosterol, TPC, TFC, and GABA).

Activation of microglia leads to the production of excessive inflammatory molecules and deleterious consequences leading to neuronal death, which has been thought to contribute to the pathogenesis of neurodegenerative diseases; cause of acute injuries (stroke, traumatic brain injury) or chronic neurodegeneration (such as Alzheimer's disease, Parkinson's disease, and chronic traumatic encephalopathy) [53]. Recent studies have reported that some compounds such as nucleoside, ergosterol, GABA, polysaccharides, and cordycepin were isolated from *Cordyceps militaris* exerted inhibited the production of NO, reduced pro-inflammatory cytokines; and they possessed an effect of neuroprotection by inhibiting microglia-mediated inflammation in LPS-induced microglia BV2 cells [35,54]. In the present study, our results demonstrated that EEOS-95 has significantly inhibited the levels of cytokines (IL-1 β , IL-6, TNF- α , and PGE2) secreted and decreased the production of NO in LPS-stimulated BV2 cells. Therefore, EEOS-95 is possible for natural ingredients to be used as therapeutics for neurodegenerative disorders with neuro-inflammation.

Peroxisome proliferator-activated receptor gamma (PPAR- γ) is a subunit of the PPAR and is a ligand-activated nuclear transcription factor; if brain injury, PPAR- γ can be "key" of cytoprotective stress responses and enhance the chances of cellular survival [53]. Furthermore, several recent studies showed that PPAR- γ is also capable of inhibiting other transcription factors, and has not only been implicated in the downregulation transcription and expression of related genes involved in proinflammatory cytokines (IL-1 β , IL-6, and TNF- α), and neuro-inflammatory genes (COX-2 and iNOS); as well as it can be inhibiting active-NF- κ B signals; playing a neuroprotective role of microglia [53,55].

Luteoloside (a flavonoid compound) significantly upregulated PPAR- γ and Nrf-2 and decreased the release of proinflammatory cytokines in focal cerebral ischemia on middle cerebral artery occlusion (MCAO) rats by inhibiting the NF- κ B pathway [56]. Ergosterol isolated from mushrooms (*Auricularia polytricha*, *Cordyceps militaris*) attenuates bisphenol A or LPS-induced BV2 microglial cell inflammation [57,58]. Zheng et al. indicated that GABA-enriched *Moringa oleifera* leaves (MLFB) fermentation broth also could effectively alleviate LPS-induced inflammatory response by inhibiting the secretions of pro-inflammatory cytokines and the anti-inflammatory activity might be related to the relatively high contents of GABA, flavonoids, phenolics and organic acids in MLFB, its mechanism might be associated with the inhibition of TLR-4/NF- κ B inflammatory signaling pathway activation [59]. Actually, treatment of EEOS-95 significantly inhibited the expression of NF- κ B, iNOS, and COX-2; also enhanced upregulated of Nrf-2 and HO-1; so, we suggested that EEOS-95, an effective activator of PPAR- γ , will be an attenuated neuro-inflammation through by modulating the PPAR- γ /Nrf-2/HO-1/NF- κ B signaling pathway, and ergosterol, GABA, flavonoids and polyphenol may be responsible for their activity.

Additionally, the nuclear factor Nrf-2 is known as another transcription factor and master regulator of detoxification and antioxidant regulation, which may play a main role in neuroprotective function [60]. Some documents suggested that it correlates with PPAR- γ and Nrf-2, they exerted against oxidative stress, which effectively reduced the inflammatory response by inhibiting NF- κ B signals [61,62]. Besides, Duan et al., pointed out the synergistic effect of PPAR- γ and Nrf-2 pathway to upregulate the expression of related genes and inhibit ferroptosis-induced neuronal injury in intracerebral hemorrhage rats *in vitro* and *in vivo* [62]. Furthermore, pro-oxidant HO-1 expression is

upregulated by oxidative stress, nitric oxide, CO, and hypoxia. Choi and colleagues also highlighted that HO-1 is present and its role in neurovascular diseases, such as age-related macular degeneration (AMD), ischemia-reperfusion injury, traumatic brain injury, and Alzheimer’s disease [63]. The abnormal HO-1 levels with Nrf-2 dysfunction are implicated in the pathogenesis of neurovascular systems related to ischemia, trauma, and aging; thus, Nrf-2/ HO-1 signal mechanism is involved in the development, oxidative stress responses, and anti-inflammation [64].

This research mainly studied the effect of EEOS-95 on PPAR- γ , an activator that inhibits oxidative stress, and against neuronal inflammation through the synergistic actions of the expression-PPAR- γ , Nrf-2, and HO-1 pathways in LPS-stimulated in the microglia BV2 cells (Figure 5). Furthermore, EEOS-95 has exhibited strong pharmacological properties and exerted a potential neuro-inflammatory effect; so, we will continue to investigate deeply explore the molecular mechanism by which PPAR- γ /Nrf-2/HO-1 pathway to neuro-protection *in vivo*, safe-dose, as well as preclinical and clinical in future studies of 95% ethanol extract of OMSC mycelia.

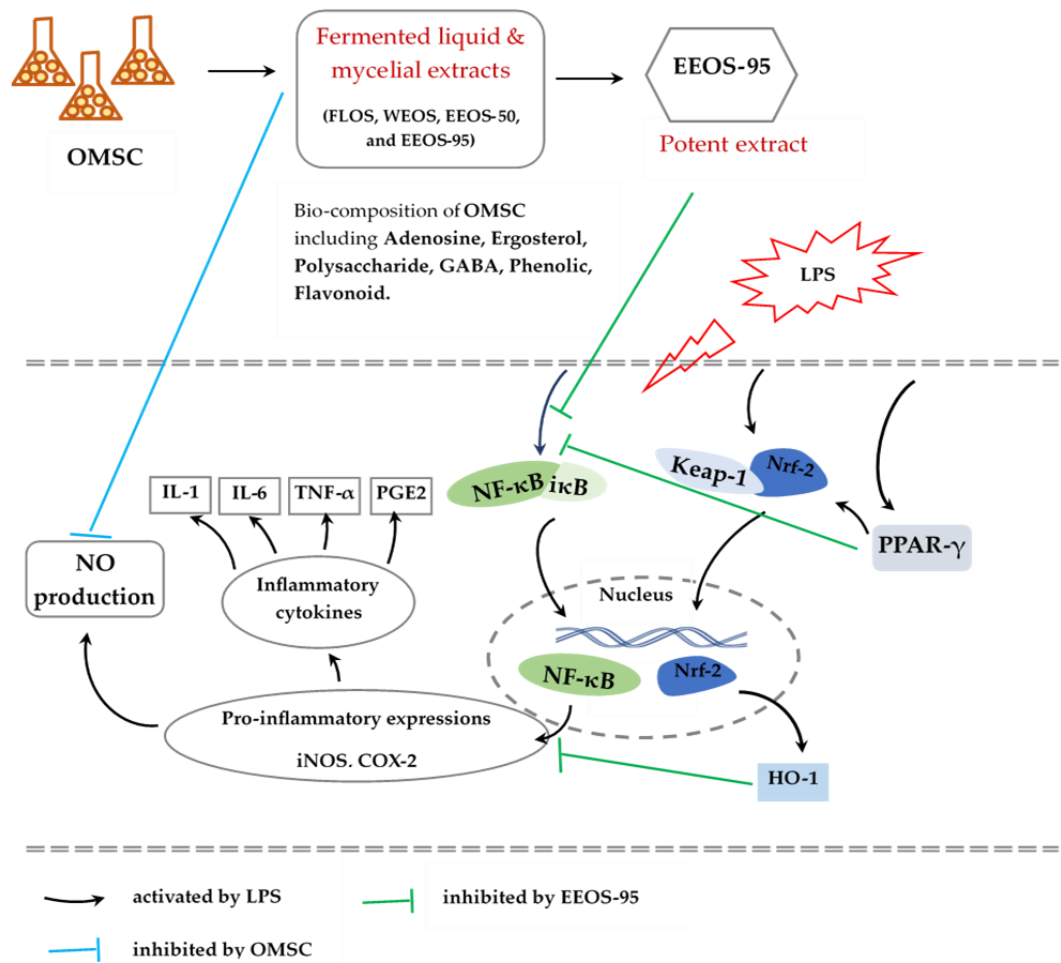


Figure 5. Schematic summaries of the effects of OMSC and EEOS-95 on lipopolysaccharide-stimulated BV2 microglial cells. FLOS: fermented liquid of OMSC, WEOS: hot water extract of OMSC, EEOS-50: 50 % ethanol extract of OMSC, EEOS-95: 95 % ethanol extract of OMSC, mycelial submerged culture of Os H101 (OMSC).

5. Conclusions

The findings demonstrate the potential of EEOS-95 to be developed into functional food due to its against neuro-inflammatory by upregulated PPAR- γ /Nrf-2/HO-1 inhibited NF- κ B/COX-2/iNOS pathways to decrease pro-inflammatory cytokines (IL-1 β , IL-6, and TNF- α). We also found six types of bioactive components involving adenosine, ergosterol, polysaccharide, GABA, TPC, and TFC

present in the extracts of OMSC. The synergistic of these phytochemicals would contribute to pharmacological properties in neuroprotection on LPS-induced BV2 microglia cells. The further identified bioactive ingredients components and proposals of possible mechanisms to obtain those bioactive compounds in the molecular mechanism will continue the further investigation. Thus, OMSC possesses a potential source of neuroprotective and also contributes to the function of food or nutraceutical pharmacy.

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