***In Vitro* Antioxidant Capacity and Neuronal Cell Toxicity of Roots of *Ostericum koreanum* Maximowicz**

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Received 3 March 2010; Accepted 21 May 2010

Abstract: *Ostericum koreanum* maximowicz (Umbelliferae), a medicinal herb in Korean Oriental Medicine, has been applied to treat cold, headache, neuralgia and arthralgia. The ethyl acetate fraction of *O. koreanum* root was subjected to *in vitro* antioxidant activity with different methods for free radical scavenging activities. In addition, the cell viability and nitric oxide release assays were performed here for the first time in neuroblastoma (Neuro-2a) cell cultures. Among all the tested methods, the ethyl acetate fraction was expressed very active, exhibiting a good Trolox equivalent values and IC₅₀, comparable to that of the commercial antioxidants, Trolox and ascorbic acid, respectively. The results showed that there was a reduction of cell viability by the fraction in a concentration dependent manner. These results suggest that *O. koreanum* shows good antioxidant activities *in vitro* by inhibiting free radicals. These findings provide a rationale for the *in vivo* testing. Also, the major constituents behind the antioxidant mechanisms of this fraction warrant further study.

Keywords: Antioxidant activity, Free radical scavenging, Neuro-2a, *Ostericum koreanum* maximowicz.

Introduction

Ostericum koreanum maximowicz (Umbelliferae) is used traditionally in Korean Oriental Medicine. The roots of *O. koreanum* have long been used to treat cold, headache, neuralgia and arthralgia in oriental medicine¹. This plant is known to have many pharmacologic activities, such as anti-tumor², anti-microbial³, antioxidant⁴, anti-inflammatory⁵ and acaricidal activity⁶. It was proposed that the components responsible for these activities were isoimperatorin, oxypeucedanin, isooxypeucedanin, oxypeucedanin bergapten, osthol, oxypeucedanin hydrate,

cnidicin, marmesinin⁷⁻⁹, 11-hydroxy-sec-O-glucosylhamaudol⁴, essential oils³ and bisabolangelone⁶. Recently, we reported that bisabolangelone isolated from *O. koreanum* inhibits the production of inflammatory mediators by down-regulation of NF- κ B and ERK MAP kinase activity in LPS-stimulated RAW264.7 cells¹⁰.

In the present study, the roots of *O. koreanum* ethyl acetate fraction was evaluated for Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide radical ($O_2^{\bullet-}$) scavenging, hydrogen peroxide (H_2O_2) scavenging, hydroxyl ($OH^{\bullet}$) radical scavenging, nitric oxide radical ($NO^{\bullet}$) scavenging and ferrous ion chelating efficacies. Moreover, the effects of ethyl acetate fraction of *O. koreanum* roots on cell viability and nitric oxide (NO) release in neuro-2a (N2a) cells were analyzed.

Experimental

1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitro blue tetrazolium salt (NBT), phenazine methosulfate (PMS), thiobarbituric acid (TBA), trichloroacetic acid (TCA), dimethyl sulphoxide (DMSO), sodium nitroprusside (SNP), sulphanilic acid, *N*-(1-Naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA), $FeSO_4 \cdot 7H_2O$, deoxyribose, 2,2'-azino-bis(3-ethylbenzthiozoline-6-sulphonic acid (ABTS), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid), a water-soluble analogue of vitamin E and 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals used were of analytical grade supplied by Fluka, Aldrich or Sigma Co.

Plant material

O. koreanum roots, cultured in Bongwha Alpine Medicinal Plant Experiment Station (BAMPES; Gyeongsangbook, Republic of Korea), were authenticated. A voucher specimen was deposited in the herbarium of BAMPES, with the registration number NK-2007-10.

Extraction and fractionation

Air-dried roots of *O. koreanum* (1.5 kg) were cut into small pieces, pulverized and extracted three times with 4.5 L of methanol at room temperature for 24 h. The supernatant was collected by filtration and the solvent was evaporated under in vacuum at 40 °C (~ 74 g). The extract (70 g) was sequentially partitioned into hexane (11 g), ethyl acetate (10 g), *n*-butanol (14 g) and water (35 g) portions. The most active ethyl acetate fraction was used for bioassays.

Antioxidant assays

In vitro antioxidant activity was studied by TEAC^{11,12}, ORAC¹³, DPPH¹⁴, superoxide radical¹⁵, hydrogen peroxide decomposition^{16,17}, hydroxyl radical¹⁸, nitric oxide radical inhibition^{19,20} and metal chelation²¹.

Cell culture and treatments

Neuro-2a (N2a; CCL-131, American Type Culture Collection, Manassas, VA) neuroblastoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep, GIBCO, Grand Island, NY). Cultures were maintained in a humidified atmosphere at 37 °C with 5% CO₂. Cells were seeded and allowed to grow for 24 h.

For treatments, 24 h of serum-starved cells were incubated with *O. koreanum* ethyl acetate fraction (2.5–30 µg/mL) for 1 h. Cell viability was measured by the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) method. The NO production was measured as the nitrite (NO₂⁻) concentration. All experiments were performed a minimum of three times and P-values lower than 0.05 was considered statistically significant.

Statistical analysis

The data are expressed as the mean ± SD of three measurements. Statistical analysis was performed using Tukey's test. $p < 0.05$ was considered statistically significant.

Results and Discussion

In this study, we designed to evaluate the antioxidant and free radical scavenging activities of ethyl acetate fraction of methanol extract of *O. koreanum* roots. From results, the *O. koreanum* roots ethyl acetate fraction showed potent antioxidant activity based on TEAC, ORAC and DPPH and other free radical scavenging tests in a dose dependent manner. The *O. koreanum* roots were found to be active in TEAC, ORAC and DPPH as 0.516, 2.884 and 1.514 mmole Trolox equivalents per grams, respectively (Table 1). The ethyl acetate fraction showed the most efficient against TEAC followed by DPPH and ORAC. From this, the *O. koreanum* was weak in scavenging the ORAC compared to others. According to the TEAC method, this fraction acted as a direct free radical scavenger, indicating that the fraction had significant antioxidant activity. The scavenging data suggests that the components within the fraction is capable of scavenging free radicals via a mechanism of electron/hydrogen donation and should be able to protect susceptible matrices from free radical-mediated oxidative degradation.

Table 1. The antioxidant activities of ethyl acetate fraction of *O. koreanum*.

	TEAC, mM TE/ g	ORAC, mM TE/ g	DPPH, mM TE/ g
<i>O. koreanum</i>	0.516 ± 0.024	2.884 ± 0.148	1.514 ± 0.011
Ascorbic acid	0.828 ± 0.010	0.030 ± 0.018	1.332 ± 0.027

Moreover, this fraction strongly scavenged O₂^{•-}, H₂O₂, OH[•] and NO[•] with the IC₅₀ being 23.582, 66.310, 170.582 and 60.779 micrograms per mL and the activity is comparable to that of ascorbic acid. The extract also strongly chelated Fe³⁺ ions at 7.846 micrograms per mL of IC₅₀ (Table 2). In the PMS-NADH-NBT system, superoxide anion, derived from dissolved oxygen from the coupling reaction of PMS-NADH, reduces NBT. Then, scavenging of H₂O₂ may be attributed to electrons donation to H₂O₂, thus neutralizing it to water. Although H₂O₂ itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to OH[•] in the cell.

Table 2. The free radical scavenging and metal chelating activities (IC₅₀ as µg/mL) of ethyl acetate fraction of *O. koreanum*

	O ₂ ^{•-}	H ₂ O ₂	OH [•]	NO [•]	Metal chelation
<i>O. koreanum</i>	23.582 ± 4.480	66.310 ± 2.653	170.582 ± 7.118	60.779 ± 1.568	7.846 ± 1.732
Ascorbic acid	8.762 ± 4.569	8.053 ± 3.677	3.034 ± 0.191	9.885 ± 0.478	43.235 ± 8.543

Values are means ± SD of three measurements. TEAC trolox equivalent antioxidant capacity, ORAC oxygen radical absorbance capacity, DPPH 1,1-diphenyl-2-picrylhydrazyl

The *O. koreanum* fraction was evaluated for their ability to scavenge $\text{OH}^\bullet$ using the deoxyribose degradation assay and nitric oxide radicals using sodium nitroprusside (SNP) as a $\text{NO}^\bullet$ donor. The chelation of ferrous ions, in which ferrozine quantitatively forms complexes with Fe^{2+} . In the presence of chelating agents, the formation of this complex is disrupted, thereby impeding the formation of the red colour imparted by the complex as well. Considering the results obtained, it may be anticipated that the ethyl acetate fraction of *O. koreanum* methanol extract have antioxidant activity, shown here by the scavenging of $\text{O}_2^{\bullet-}$, H_2O_2 , $\text{OH}^\bullet$ and $\text{NO}^\bullet$ and ferrous ion chelating efficacies.

To determine the effects of roots of ethyl acetate fraction of *O. koreanum* on cell viability, the N2a cells were exposed to *O. koreanum* (2.5 ~ 30 $\mu\text{g/mL}$) for an incubation time of 1 h. In Figure 1, the MTT test after 1 h of incubation with *O. koreanum* roots does not indicate any significant viability difference at 2.5 and 5.0 micrograms per mL in treated N2a cell cultures in comparison to control. A significant decreasing of viability is observed in roots of *O. koreanum* ethyl acetate fraction at 7.5 ~ 30 micrograms per mL treated N2a cells in comparison to control. NO determination was performed after 1 h of incubation in the presence of *O. koreanum* (2.5 ~ 30 $\mu\text{g/mL}$). The treatment with 7.5 micrograms per mL of *O. koreanum* ethyl acetate fraction produces NO decrease significantly when compared to control, but other doses did not show any significant changes in NO release. From this result that the minimal doses of *O. koreanum* have no toxicity effects.

Figure 1. Effect of *O. koreanum* on cell viability and nitric oxide release in neuro-2a cells. Values are means $\pm$ SD of three measurements. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA/Tukey) compared with untreated normal

Conclusion

This study demonstrates that the various free radical scavenging mechanisms of *O. koreanum* ethyl acetate fraction may be attributed to its strong abilities as a hydrogen donor. Also, the study verified the effectiveness of *O. koreanum* on cell viability and nitric oxide release in cell culture model. As a result, the medicinal claims of *O. koreanum* being used in the treatment of various diseases may be in part due to the antioxidant activity having potential in scavenging of free radicals come out for the therapeutic value. Further isolation of antioxidant constituents and in vivo antioxidant studies are warranted.

Acknowledgments

This study was supported by the research fund of Studies on the Development of Oriental Medicine for Allergic Rhinitis from Ministry of Health, Welfare & Family Affairs (2009), Republic of Korea (to Y.-K. Park).

References

- 1 Ryu K S and Yook C S, *J Pharm Soc Korea*, 1968, **12**, 59-64.
- 2 Kang T J, Lee S Y, Singh R P, Agarwal R and Yim D S, *Acta Oncol.*, 2009, **48**, 895-900.
- 3 Shin S, *Arch Pharm Res.*, 2005, **28**(7), 765-769.
- 4 Park Y J, Kim H J, Lee S J, Choi H Y, Jin C and Lee Y S, *Chem Pharm Bull.*, 2007, **55**(7), 1065-1066.
- 5 Park H J, Bae G S, Kim D Y, Seo S W, Park K B, Kim B J, Song J M, Lee K Y, Na C, Shin B C, Park S J, Song H J and Hwang S Y, *Kor J Herbology*, 2008, **23**, 127.
- 6 Lee C K and Woo W S, *Kor J Pharmacogn.*, 1982, **13**, 10-13.
- 7 Kwon Y S, In K K and Kim C M, *Kor J Pharmacogn.*, 2000, **31**, 284-287.
- 8 Ryu S Y, Kou N Y, Choi H S, Ryu H, Kim T S and Kim K M, *Planta Med.*, 2001, **67**, 172-174.
- 9 Kang S W, Kim H K, Lee W J and Ahn Y J, *J Agric Food Chem.*, 2006, **54**, 3547-3550.
- 10 Jung H W, Mahesh R, Park J H, Boo Y C, Park K M and Park Y K, *Int Immunopharmacol.*, 2010, **10**, 155-162.
- 11 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, *Free Radic Biol Med.*, 1999, **26**, 1231-1237.
- 12 Schlesier K, Harwat M, Böhm V and Bitsch R, *Free Radical Res.*, 2002, **36**, 177-187.
- 13 Cao G, Verdon C P, Wu A H B, Wang H and Prior R L, *Clin Chem.*, 1995, **41**, 1738-1744.
- 14 Gyamfi M A, Yonamine M and Aniya Y, *Gen Pharmacol.*, 1999, **32**, 661-667.
- 15 Robak J and Gryglewski R J, *Biochem Pharmacol.*, 1988, **36**, 317.
- 16 Sinha A K, *Anal Biochem.*, 1972, **47**, 389-394.
- 17 Ohkawa H, Ohishi N and Yagi K, *Anal Biochem.*, 1979, **95**, 351-358.
- 18 Chung S K, Osawa T and Kawakishi S, *Biosci Biotech Biochem.*, 1997, **61**, 118-124.
- 19 Garratt D C, *The quantitative analysis of drugs*; Chapman and Hall: Japan, 1964, **3**, 456-458.
- 20 Marcocci P L, Sckaki A and Albert G M, *Methods Enzymol.*, 1994, **234**, 462-475.
- 21 Decker E A and Welch B, *J Agric Food Chem.*, 1990, **38**, 674.