

Immunobiological Activity of a New Benzyl Benzoate from the Aerial Parts of *Solidago virga-aurea* var. *gigantea*

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The chromatographic separation of the hexane soluble fraction of the methanol extract of the aerial parts of *Solidago virga-aurea* var. *gigantea* M₁₀. (Compositae) led to the isolation of a new benzylbenzoate (1) together with four known benzylbenzoates (2~5). Their structures were determined as 2-methoxybenzyl-2-hydroxybenzoate (1), benzyl-2-hydroxy-6-methoxybenzoate (2), 2-methoxybenzyl-2,6-dimethoxybenzoate (3), 2-methoxybenzyl-2-methoxy-6-hydroxybenzoate (4), and benzyl-2,6-dimethoxybenzoate (5). Their structures were established by spectroscopic methods. Biological effects of compounds, 1 and 2, were investigated *in vitro* using mouse peritoneal macrophages. The benzylbenzoates (1 and 2) could serve as immunotherapeutic agents by stimulating macrophage functions, with potential use in the treatment of infectious diseases.

Key words: *Solidago virga-aurea* var. *gigantea* M₁₀, Compositae, Benzyl benzoates, Macrophage

INTRODUCTION

Solidago virga-aurea var. *gigantea* M₁₀. (Compositae), a perennial herb, is mainly distributed in the southern island of South Korea and especially cultivated as culinary vegetable in the Ullung island. This plant has been used as stomachic and diuretic in Korean folk medicine (Lee, 1979). Previous phytochemical studies demonstrated the presences of erythrodiol-3-acetate, α -tocopherol-quinone, *trans*-phytol and 2-methoxybenzyl-2,6-dimethoxy benzoate in the hexane-soluble fraction of this plant (Sung *et al.*, 1999). As part of our ongoing studies on biologically active substances of the genus *Solidago* for Korean Compositae plants, we have investigated chemical components of the methanol extract of the aerial parts of *Solidago virga-aurea* var. *gigantea*. A new 2-methoxybenzyl-2-hydroxybenzoate (1) and four known benzylbenzoates (2~5), benzyl-2-hydroxy-6-methoxybenzoate (2), 2-methoxybenzyl-2,6-dimethoxybenzoate (3), 2-methoxybenzyl-2-methoxy-6-hydroxybenzoate (4), and benzyl-2,6-dimeth-

oxybenzoate (5) were isolated from the hexanesoluble fraction of the methanol extract of this plant. This paper describes the isolation and structural characterization of these compounds and their *in vitro* biological effects on some parameters of mouse macrophage functions.

MATERIALS AND METHODS

General experimental procedure

Mps: uncorr. NMR: Bruker AMX 500 and Varian UNITY INOVA 500. IR: in CCl₄, Nicolet model 205 FT-IR spectrophotometer. MS: VG70-VSEQ mass spectrometer. Column chromatography : Silica gel 60 (Merck, 70230 mesh and 230~400 mesh), Licroprep. RP-18 (Merck) and Sephadex LH-20. TLC: Merck precoated Si gel F₂₅₄ plates and RP-18 F_{254s} plates. LPLC: Merck Lichroprep Lobar[®]-A Si 60 (240×10 mm)

Plant materials

The aerial parts of *Solidago virga-aurea* var. *gigantea* (Compositae) were collected at Ullung island, Korea in August, 2001. A voucher specimen (SKK-01-014) was deposited at the College of Pharmacy in SungKyunKwan University.

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Extraction, separation and purification of compounds

The aerial parts of *Solidago virga-aurea* var. *gigantea* (2 kg) were extracted with MeOH (10 L) five times at room temperature. The resulting methanol extract (190 g) was suspended in H₂O and successively partitioned to give *n*-hexane (60 g), CH₂Cl₂ (2.8 g), EtOAc (9 g) and BuOH (26 g). The *n*-hexane extract (60 g) was chromatographed on silica gel using gradient solvent system of *n*-hexane:EtOAc (10:00:1) to give five main fractions [Fr.S-1 (7 g), Fr.S-2 (3.5 g), Fr.S-3 (7.5 g), Fr.S-4 (8 g), Fr.S-5 (18 g)]. Fr.S-2 (3.5 g) was chromatographed on silica gel (250 g) eluted with *n*-hexane:EtOAc (6:1) to give four subfractions (S-21~S-24). Fr.S-22 (600 mg) was purified with Sephadex LH-20 (CH₂Cl₂:MeOH=1:1) and RP Lobar[®]-A column (85% MeCN) to yield 2-methoxybenzyl-2-hydroxy benzoate (**1**; 5 mg) and benzyl-2-hydroxy-6-methoxybenzoate (**2**; 25 mg). Fr.S-24 (800 mg) was chromatographed on silica gel (250 g) eluted with *n*-hexane:EtOAc (7:1) and purified with Sephadex LH-20 (CH₂Cl₂:MeOH=1:1) to yield 2-methoxybenzyl-2,6-dimethoxybenzoate (**3**; 150 mg). Fr.S-3 (7.5 g) was chromatographed on silica gel (250 g) eluted with *n*-hexane:EtOH (3:1) to give five subfractions (S31~S35). Fr.S-35 (900 mg) was purified with Sephadex LH-20 (CH₂Cl₂:MeOH=1:1) to yield 2-methoxybenzyl-2-methoxy-6-hydroxybenzoate (**4**; 250 mg). Fr.S33 (1.4 g) was chromatographed on Sephadex LH-20 (CH₂Cl₂:MeOH=1:1) to give five subfractions (S331~S335). The second subfraction (230 mg) was purified with RP Lobar[®]-A column (85% MeCN) to yield benzyl-2,6-dimethoxybenzoate (**5**; 100 mg).

2-Methoxybenzyl-2-hydroxybenzoate (**1**)

White powder, mp 103°C; IR (CHCl₃) ν_{\max}^{neat} ν =3100, 1665, 1260; EI-MS *m/z* (ret. int) : 258 (M⁺, 12), 242 (33), 137 (58), 121 (30), 91 (100), 77 (40), 65 (17), 51 (11); HR-EI-MS *m/z* : 258.0906 [M]⁺ (Calcd for C₁₅H₁₄O₄: 258.0892); ¹H-NMR (500 MHz, CDCl₃) : δ 3.86 (3H, s, 2'-OCH₃), 5.39 (2H, s, H-8), 6.92 (H, dd, *J* = 8.0, 2.0 Hz, H-3), 6.97 (H, dd, *J* = 8.0, 2.0 Hz, H-5), 7.31 (H, m, H-4), 7.31 (H, br.d, *J* = 8.5 Hz, H-3'), 7.41 (H, m, H-5'), 7.44 (H, m, H-6), 7.55 (H, br.t, *J* = 7.5 Hz, H-4'), 8.08 (H, dd, *J* = 8.5, 1.5 Hz, H-4'); ¹³C-NMR (125 MHz, CDCl₃) : Table I.

Benzyl-2-hydroxy-6-methoxybenzoate (**2**)

Colorless oil, IR (CHCl₃) ν_{\max}^{neat} ν =3150, 1680, 1270; EI-MS *m/z* (ret. int) : 258 (M⁺, 46), 150 (44), 107 (8), 91 (100), 65 (12), 51 (3); ¹H-NMR (500 MHz, CDCl₃) : δ 3.86 (3H, s, 2-OCH₃), 5.43 (2H, s, H-8), 6.42 (2H, dd, *J* = 8.5, 0.5 Hz, H-5), 6.60 (H, dd, *J* = 8.5, 0.5 Hz, H-3), 7.34 (H, dd, *J* = 8.5, 8.0 Hz, H-4), 7.35 (H, t, *J* = 7.5 Hz, H-4'), 7.40 (2H, td, *J* = 7.5, 1.5 Hz, H-3', H-5'), 7.48 (2H, d, *J* = 7.5 Hz, H-2', H-6'); ¹³C-NMR (125 MHz, CDCl₃) : Table I.

Table I. ¹³C-NMR Spectral data of Compounds 1-5 (CDCl₃, 125 MHz, δ ppm)

C	1	2	3	4	5
1	120.7	127.7	124.7	124.1	125.0
2	157.8	161.4	157.7	163.2	157.7
3	110.7	102.6	104.2	103.6	104.2
4	133.1	136.0	131.4	134.9	136.4
5	124.7	103.5	104.2	102.4	104.2
6	129.7	163.9	157.7	161.0	157.7
7	166.8	171.2	166.8	170.2	166.8
8	67.2	67.1	62.5	62.7	60.7
1'	124.1	110.3	113.5	110.0	113.2
2'	156.9	128.8	157.5	156.9	128.6
3'	112.6	128.7	110.5	110.2	128.3
4'	129.9	135.5	129.4	128.5	131.4
5'	128.5	128.3	120.6	120.4	128.3
6'	130.7	128.7	129.4	129.1	128.4
2-OCH ₃		56.4	56.2	56.1	56.2
6-OCH ₃			56.2		56.2
2'-OCH ₃	55.7		55.6	55.3	

2-Methoxybenzyl-2,6-dimethoxybenzoate (**3**)

White powder, mp 100°C; IR (CHCl₃) ν_{\max}^{neat} ν =1725, 1265; EI-MS *m/z* (ret. int) : 302 (M⁺, 13), 165 (79), 138 (67), 121 (64), 91 (100), 77 (44), 65 (22), 51 (17); ¹H-NMR (CDCl₃, 500 MHz) : δ 3.81 (6H, s, 2,6-OCH₃), 3.85 (3H, s, 2'-OCH₃), 5.51 (2H, s, H-8), 6.56 (2H, d, *J* = 8.0 Hz, H-3, H-5), 6.89 (H, d, *J* = 8.5 Hz, H-3'), 6.99 (H, t, *J* = 7.5 Hz, H-5'), 7.28 (H, t, *J* = 8.5 Hz, H-4), 7.30 (H, td, *J* = 8.5, 1.5 Hz, H-4'), 7.52 (H, d, *J* = 7.5 Hz, H-6'); ¹³C-NMR (CDCl₃, 125 MHz) : Table I.

2-Methoxybenzyl-2-methoxy-6-hydroxybenzoate (**4**)

White powder, mp 99°C; IR (CHCl₃) ν_{\max}^{neat} ν =3300, 1720, 1260; EI-MS *m/z* (ret. int) : 288 (M⁺, 89), 151 (8), 121 (100), 91 (72), 65 (11), 51 (6); ¹H-NMR (500 MHz, CDCl₃) : δ 3.87 (3H, s, 2-OCH₃), 3.88 (3H, s, 2'-OCH₃), 5.48 (2H, s, H-8), 6.43 (H, d, *J* = 8.5 Hz, H-5), 6.60 (H, d, *J* = 8.5 Hz, H-3), 6.91 (H, d, *J* = 8.5 Hz, H-3') 6.99 (H, t, *J* = 7.5 Hz, H-5'), 7.32 (H, t, *J* = 8.5 Hz, H-4), 7.34 (H, td, *J* = 8.5, 1.5 Hz, H-4'), 7.54 (H, dd, *J* = 7.5, 1.0 Hz, H-6'); ¹³C-NMR (125 MHz, CDCl₃) : Table I.

Benzyl-2,6-dimethoxybenzoate (**5**)

White powder, mp 69°C; IR (CHCl₃) ν_{\max}^{neat} ν =1715, 1255; EI-MS *m/z* (ret. int) : 272 (M⁺, 22), 181 (3), 165 (100), 91 (99), 77 (52), 65 (37), 51 (24); ¹H-NMR (500 MHz, CDCl₃) : 3.81 (6H, s, 2,6-OCH₃), 5.41 (2H, s, H-8), 6.56 (2H, d, *J* = 8.5 Hz, H-3, H-5), 7.30 (H, t, *J* = 8.5 Hz, H-4), 7.34 (H, m, H-4'), 7.38 (2H, m, H-3', H-5'), 7.48 (H,

dd, $J = 8.5, 1.5$ Hz, H-2', H-6"); ^{13}C -NMR (125 MHz, CDCl_3): Table I.

Isolation of inflammatory peritoneal macrophages

Thioglycollate-elicited peritoneal exudate cells were obtained from 6-8 week-old C57BL/6 male mice (Charles River Breeding Laboratories, Atsugi, Japan) following intraperitoneal injection of 1 mL Brewer Thioglycollate broth (4.05 g/100 mL) (Difco Laboratories, Detroit, MI) and lavage of the peritoneal cavity with 5 mL of medium 3-4 days later. The cells were washed twice and resuspended in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 IU/mL) and streptomycin (100 mg/mL) (RPMI-FBS). Macrophages were isolated from peritoneal exudate cells as described by Moon and Pyo (2000). Peritoneal exudate cells were seeded at densities of $5\text{-}6 \times 10^5$ cells/cm² on teflon-coated petri dishes (100×15 mm) and the macrophages were allowed to adhere for 2-3 h in 5% CO₂ humidified atmosphere. Teflon-coated petri dishes were prepared by spraying with aerosolized teflon (Fisher Scientific, Pittsburgh, PA) and sterilizing with ultraviolet light for 3 h. The nonadherent cells were removed by washing the dishes twice with 10 mL prewarmed medium and dishes were incubated for 10 min at 4°C. The supernatants were then carefully removed and discarded and the plates were washed once with prewarmed Dulbecco's Phosphate Buffered Saline (PBS) (GIBCO). Cold PBS (15 mL) containing 1.5% FBS (PBS-FBS) was added followed by 0.3 mL of 0.1 M EDTA (pH 7.0). The plates were incubated for 15 min at room temperature and the macrophages removed by rinsing 10 times using a 10 mL syringe. The viability of the detached cells was assessed by trypan blue exclusion and proportion of macrophages determined after cytoplasmic staining with acridine orange and examination using a fluorescence microscope. Cell preparations were >95% viable and contained >95% macrophages.

Treatment of peritoneal macrophages with compounds

Peritoneal macrophages were seeded at a concentration of 1.0×10^5 cells/well in 96-well microplate plates (Costar, Cambridge, MA, USA). Nonadherent cells were removed after 2 h at 37°C by washing twice with RPMI-FBS. The necessary assays were performed to measure the effects of compounds on macrophage functions as described by Moon *et al.* (1999). Before dilution in medium, appropriate amounts of compounds **1** and **2** were dissolved in dimethylsulphoxide (DMSO) and stock solutions were prepared in RPMI-1640 medium (Life Technologies, Grand Island, NY). They were sterile filtered using nonpyrogenic 0.22 μm filters (Costar), used fresh or kept no longer than 2 weeks at -20°C. Desired working

concentrations were prepared by diluting the stock solution in RPMI-1640 medium. The total amount of DMSO did not exceed 0.5% upon testing, an amount which was considered of no significance in the assays used. Compounds **3**, **4**, and **5** were not tested for biological activities because of the solubility. All tissue culture reagents, the thioglycollate broth and compounds were assayed for endotoxin contamination by the Limulus lysate test (E-Toxate, Sigma, St Louis, MO) and found to be less than 10 pg/mL.

Cytotoxicity evaluation

Compounds **1** and **2** at concentrations presented herein were shown to be non-toxic by the following procedure (Son *et al.*, 2001). Viability of cells treated with compounds or supernatant from macrophages treated with compounds was determined using MTT assay. Cells were treated with or without various doses of compounds in quadruplicate for indicated time and subsequently 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) was added for 4 h. In addition, a visual inspection of the cells was performed by trypan blue exclusion staining under inverted microscope.

Macrophage-mediated cytotoxicity

The assay for macrophage cytotoxicity was performed by modification of the technique described previously (Flick and Gifford, 1984; Moon *et al.*, 1999). Briefly, macrophages (1.0×10^5 cells/well) were seeded in 96 well microplates and co-incubated with B16 melanoma cells (1.0×10^4 /wells: an initial effector:target cell ratio of 10:1) in the presence of various doses of compounds for 20 h at 37°C in a 5% CO₂ incubator. Cell density was then assessed by incubating the cells with 25 g/mL MTT for another 4 h. Formazan produced was dissolved in dimethyl sulfoxide and the optical density of each well at 540 nm was determined using a Molecular device microplate reader (Menlo Park, CA). Cytolytic activity is expressed as the percentage of tumor cytotoxicity where:

$$\% \text{ cytotoxicity} = \left\{ 1 - \frac{\text{OD of (macrophages+target cells)} - \text{OD of macrophages}}{\text{OD of target cells}} \right\} \times 100$$

TNF-α determination by ELISA

The concentration of TNF-α production in culture supernatants was determined by the use of ELISA kits (Endogen, Woburn, MA) according to the manufacturers protocol.

Statistical analysis

The significance of differences between control and treated test was analyzed using Students *t*-test. Differences

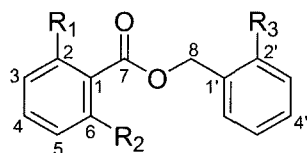
were considered significant at $P < 0.05$ and significant values were represented by an asterisk.

RESULTS AND DISCUSSION

Compound **1** was obtained as a white powder and its molecular formula was determined to be $C_{15}H_{14}O_4$ by HREIMS (m/z 258.0906, $[M]^+$). Its IR spectrum indicated the presence of a hydroxy group at 3100 cm^{-1} and an ester group at 1665 and 1260 cm^{-1} . The $^1\text{H-NMR}$ spectrum showed the signals of a methoxy group at δ 3.86 (3H, s, 2'-OCH₃) and an oxygenated methylene group at δ 5.39 (2H, s, H-8). Eight aromatic protons displayed signals in the region of δ 6.92 to 8.08. The $^{13}\text{C-NMR}$ spectrum showed 15 signals composed of a methoxy group (δ 55.7), an oxygenated carbon (δ 67.2), 12 aromatic carbons and a carbonyl carbon (δ 166.8). Based on the above mentioned evidences, the structure of **1** was presumed to be a benzyl benzoate with a hydroxy and a methoxy group. The positions of the hydroxy and the methoxy group (δ 55.7) were confirmed by HMBC data which showed the correlation of H-3 to C-2 and the correlation of H-8 (δ 5.39) and H-3' (δ 7.31) to methoxy carbon, respectively (Fig. 2). The analysis of $^1\text{H-}^1\text{H}$ COSY, HMQC and HMBC spectra were allowed to assign all proton and carbon signals in the structure of **1**. Thus, the new structure of **1** was determined as 2-methoxybenzyl-2-hydroxybenzoate.

Benzyl-2-hydroxy-6-methoxybenzoate (**2**) (Kodpinid *et al.*, 1984), 2-methoxybenzyl-2,6-dimethoxybenzoate (**3**) (Sung *et al.*, 1999), 2-methoxybenzyl-2-methoxy-6-hydroxybenzoate (**4**) (Anthonsen and Gudrun 1971) and benzyl-2,6-dimethoxybenzoate (**5**) (Lu *et al.*, 1993; Singh *et al.*, 1998) were characterized by comparing their physical and spectroscopic data with those of reported literatures.

The effect of compounds, **1** and **2**, was evaluated for tumoricidal activity *in vitro* of macrophages against B16 tumor cells. Thioglycollate-elicited macrophages were cocultured with B16 tumor cells in the presence of various



- | | | | |
|----------|----------------------|----------------------|----------------------|
| 1 | $R_1 = \text{OH}$ | $R_2 = \text{H}$ | $R_3 = \text{OCH}_3$ |
| 2 | $R_1 = \text{OH}$ | $R_2 = \text{OCH}_3$ | $R_3 = \text{H}$ |
| 3 | $R_1 = \text{OCH}_3$ | $R_2 = \text{OCH}_3$ | $R_3 = \text{OCH}_3$ |
| 4 | $R_1 = \text{OCH}_3$ | $R_2 = \text{OH}$ | $R_3 = \text{OCH}_3$ |
| 5 | $R_1 = \text{OCH}_3$ | $R_2 = \text{OCH}_3$ | $R_3 = \text{H}$ |

Fig. 1. Structures of Compounds 1-5

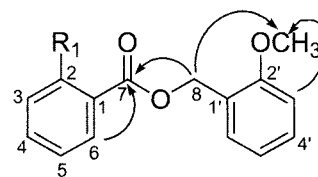


Fig. 2. Selected HMBC correlations of compound **1**

doses (1-100 $\mu\text{g/mL}$) of compounds, **1** and **2**, for 20 h. B16 tumor cells were used as targets since they are either TNF- α or NO sensitive. The time of incubation and concentration of compounds used in these experiments had no effect on the viability as determined by MTT assay (Fig. 3), trypan blue staining and morphology of the endothelial cells (data not shown). Compound **1** significantly induced tumoricidal activity of macrophage at concentration of 1 $\mu\text{g/mL}$, while tumoricidal activity of macrophage was slightly decreased at concentrations of 10 and 100

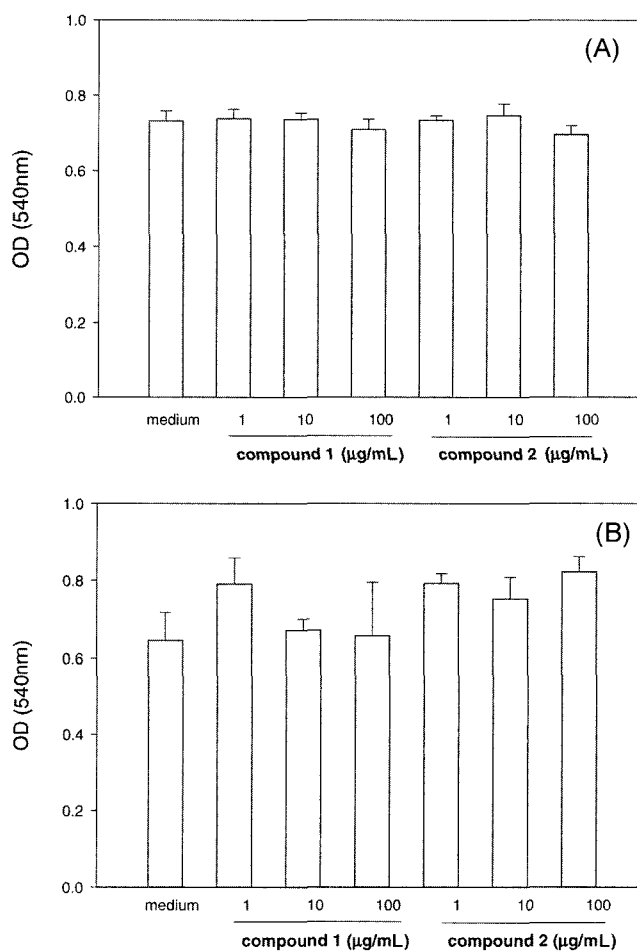


Fig. 3. Effect of compounds on cell viability of macrophage (A) and B16 (B). Cells were treated with various doses of compounds for 20 hr and cellular proliferation was then determined by the MTT assay. Each experiment was performed in quadruplicate. The results are mean \pm S.E.M from three independent experiments.

mg/mL (Fig 4A). Compound 2 increased the cytotoxicity by macrophages in a dose-dependent manner (Fig 4B). These data suggest that compound 2 is slightly more effective than compound 1.

Once activated, activated macrophages produce a large number of cytotoxic molecules (Sone and Key, 1986; Hibbs et al., 1987). We next examined the ability of compounds 1 and 2 to induce NO and TNF- α , which are currently believed to be the primary species involved killing tumor cells. Compounds were observed to activate TNF- α production by peritoneal macrophages. We found that pretreatment with compounds significantly stimulated TNF- α production by inflammatory macrophages compared with the response of untreated macrophages (Fig. 5). However, compounds 1 and 2 did not enhance production

of NO (data not shown). These results implicate that TNF- α may be a major factor in the tumoricidal activity of compounds-exposed macrophages. Additional support for the conclusion that tumoricidal activity of compounds 1 and 2 was a result of its production of TNF- α came from the observation that culture supernatant from macrophage treated with compounds was able to kill B16 cells (Fig. 6). In addition, the results did not totally rule out the possibility that reactive oxygen intermediates (ROI) are involved in tumoricidal activity induced by these compounds, because ROI has been known to be cytotoxic molecules produced by activated macrophages (Keller et al, 1999).

The benzylbenzoate (1 and 2) isolated from *Solidago virga-aurea* var. *gigantea* M₁₀ possess immunostimulatory activities, suggesting that these compounds might become candidates for antitumoral therapy.

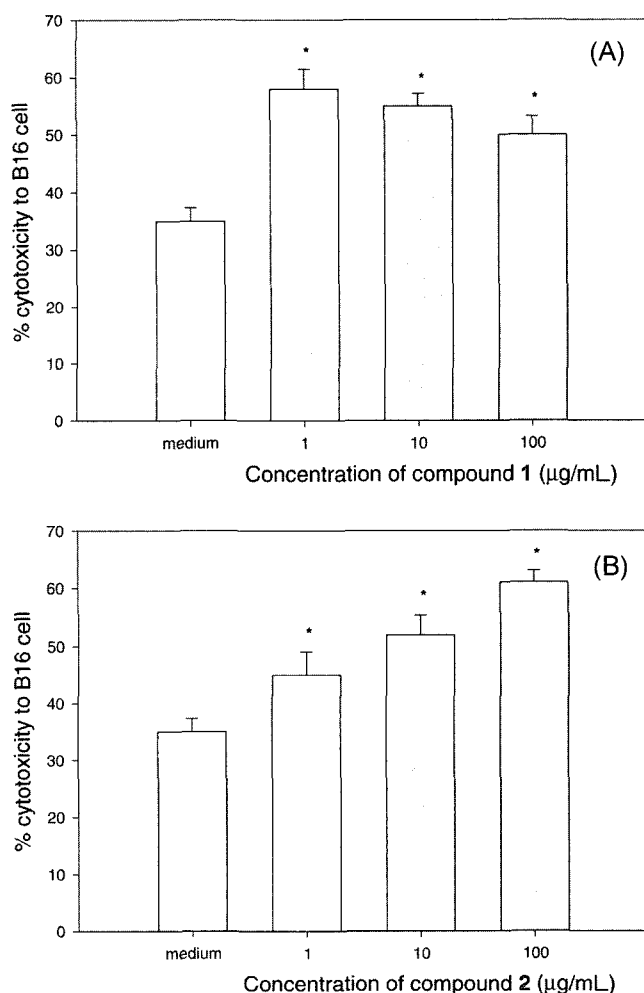


Fig. 4. Tumoricidal activities of compounds-treated murine peritoneal macrophages against B16 melanoma cells. Macrophages were treated with various doses of compounds for 20 h. Tumoricidal activity was determined as described in materials and methods at an initial effector/target ratio of 10:1. The results are mean \pm SEM of quintuplicates from one representative experiment of three. * p <0.05; significantly different from control (medium treated).

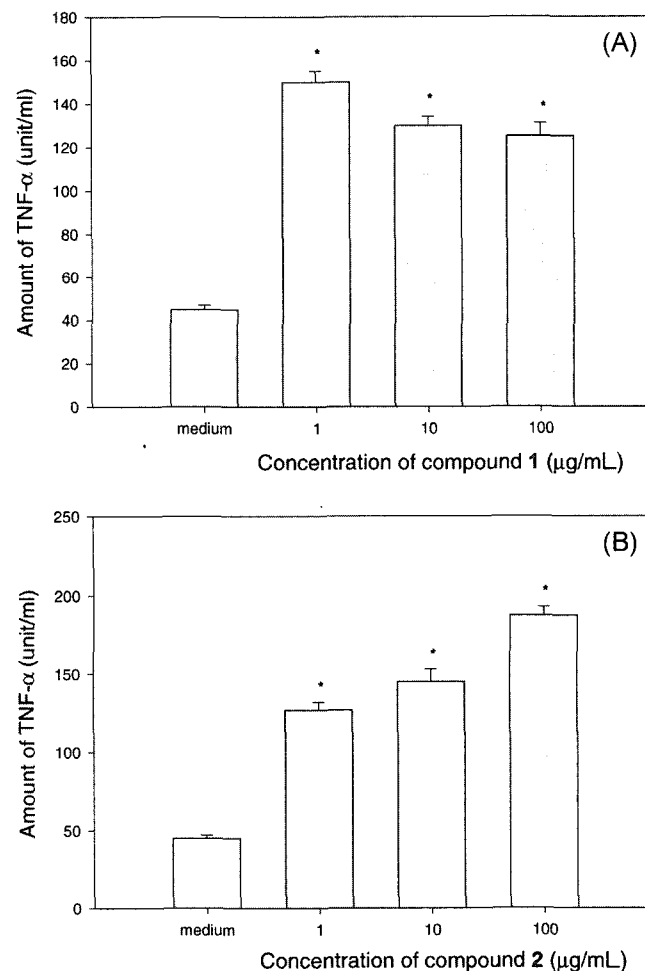


Fig. 5. TNF- α production from peritoneal macrophages stimulated with compounds. Macrophages were treated with compounds for 20 h. Culture supernatants were collected and the level of TNF- α was measured as described in materials and methods. The results are mean \pm SEM of quintuplicates from one representative experiment of three. * p <0.05; significantly different from control (medium treated).

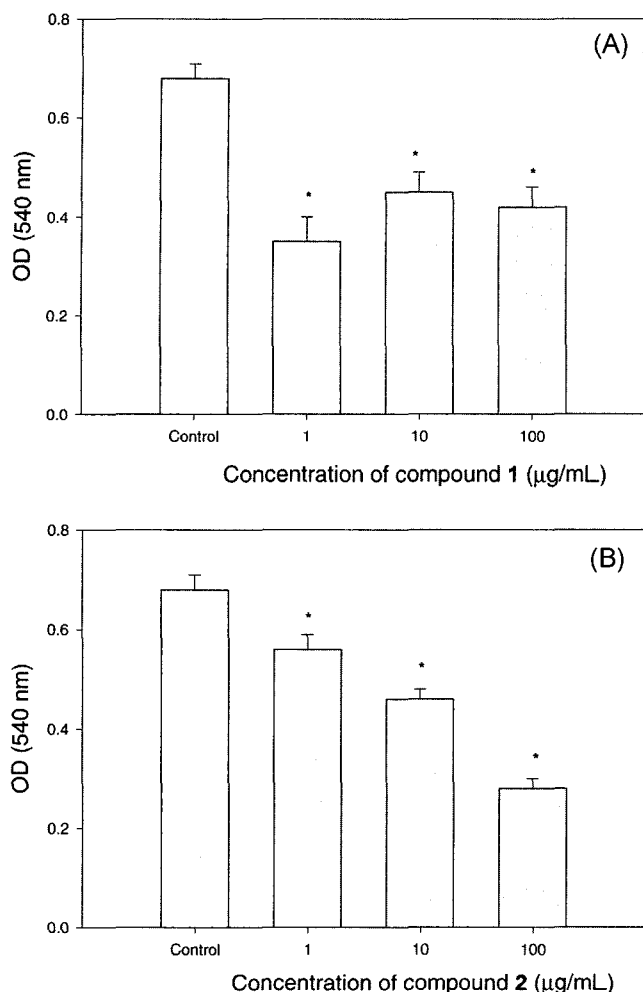


Fig. 6. Cytotoxicity of supernatant from macrophages treated with compounds. Macrophages were treated with compounds for 20 h. Culture supernatants were collected and their cytotoxicity was measured as described in materials and methods. The results are mean SEM of quintuplicates from one representative experiment of two. * $p < 0.05$; significantly different from control (medium treated).

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