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Inhibitory Effects of *Artemisia fukudo* Makino Extracts for Nitric Oxide Generation in LPS- and Interferon-γ-stimulated RAW 264.7 Cells

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갯쑥(*Artemisia fukudo* Makino) 추출물의 LPS와 Interferon-γ로 자극한 RAW264.7 세포주의 NO 생성 저해 효과

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Abstract

갯쑥(***Artemisia fukudo* Makino,** 일명 바다쑥)은 바닷가 염전 주변 습지에서 자라는 염생식물로 어린 갯쑥은 나물로 먹어왔으며, 민간에서 간에 좋다고 알려져 있다. 본 연구에서는 염생식물의 기능 성분을 탐색하는 연구의 일환으로 마우스유래 대식세포주인 **RAW264.7** 세포를 이용하여 갯쑥의 **nitric oxide(NO)** 생성 억제 작용을 알아보고자 하였다. 갯쑥은 **methanol** 추출과 열수 추출한 후 농축하여 실험하였고, **methanol** 추출물은 다시 **hexane, chloroform, ethylacetate, butanol, water**로 순차 용매 분획하여 농축하여, 각각의 추출물을 **RAW264.7** 세포에 농도별 분획별로 처리한 후 **LPS**와 **interferon-γ**로 자극한 **RAW 264.7** 세포주의 **NO** 생성을 측정하여 **NO** 생성 저해율을 구하였다. 갯쑥 **methanol** 추출물의 높은 **NO** 생성 저해율과 **iNOS** 활성 저해율을 나타내었으며**(IR=90.7%**과 **IR=81.6%),** 각각의 추출물중 분획물이 **100 μg/mL** 농도에서 **hexane** 분획물이 가장 높은 **NO** 생성 저해 효과**(IR=92.1%)**와 **iNOS** 활성 억제 효과**(IR=85.0%)**를 나타내었다. 갯쑥의 **methanol** 추출물, **hexane**과 **chloroform** 분획물에서 높은 **NO** 생성 저해 효과를 나타내어 새로운 암 예방인자 물질로 기대되며, 따라서 본 연구 결과는 암 예방인자 분리를 위한 기초 자료가 될 것이며, 나아가 성분 분리 연구와 동물 실험을 통한 기전 연구가 수행되어야 할 것이다.

Key words : *Artemisia fukudo* Makino, nitric oxide generation, RAW 264.7 cells, chemoprevention, halophyte.

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are continuously formed as a result of normal cellular functions, pathological process and toxic exposure during a cell's life (Halliwell & Gutteridge 1990).

Nitric oxide (NO) is generated by a family of isoforms of NO synthase (NOS) in several cell lines (Ignarro *et al* 1993). There are at least two types of distinguishable NOS. One is a constitutive isoform (cNOS) which is subdivided into neuronal (nNOS) and endothelial (eNOS) NOS, and is calcium-dependent. Continuous release of NO by cNOS keeps the vasculature in an active state of vasodilation, which is of paramount importance for the maintenance of normal blood pressure. Another is an inducible isoform (iNOS). Endotoxin and a number of cytokines, including interferon and interleukin, bring about the expression of inducible NOS (iNOS) in macrophages.

NO is known to cause mutagenesis (Arroyo *et al* 1992) and deamination of DNA bases (Wink et al 1991) and to play an important role in the formation of carcinogenic N-nitroso compounds *in vivo* (Miwa *et al* 1987, Grisham *et al* 1992, Wu *et al* 1993). NO rapidly and spontaneously reacts with triplet oxygen ($^3O_2$) to form stable anions, nitrite and nitrate (Ignarro *et al* 1993, Tayeh & Marletta 1989). They will be non-enzymatically decompose N-nitrosate primary and secondary amines to produce carcinogenic nitrosamines (Oshima *et al* 1991, Grisham *et al* 1992).

The anion activates the constitutive and inducible forms of cyclooxygenase (COX-1 and COX-2, respectively), which are rate-determining enzymes for prostaglandin biosynthesis during the inflammatory process (Salvemini *et al* 1993, Landino *et al* 1996, Kim *et al* 2008). On the basis of these lines of

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evidence, we assume that iNOS inhibition is a new approach for cancer chemoprevention.

iNOS-induced NO generation is known to be connected with the conversion of L-arginine to L-citrulline (Kerwin & Heller 1994, Hibbs *et al* 1987, Szabo *et al* 1993). They inhibit NO generation in activated mouse macrophages as a result of iNOS inhibition (Hansan *et al* 1993, McCall *et al* 1991, Misko *et al* 1993, Baydoun *et al* 1994). Ohata *et al* (1997) found that the ω-3 polyunsaturated fatty acids (PUFAs), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and α-linolenic acid, suppressed NO generation in lipopolysaccharide (LPS)-stimulated macrophages by inhibiting the induction of iNOS mRNA expression. This may partly be an action mechanism of cancer preventive effects of ω-3 PUFAs.

On the other hand, there is now ample evidence that excess and/or prolonged generation of nitric oxide (NO) causes carcinogenesis in digestive organs (Goto *et al* 1999, Ambs *et al* 1988). NO is drastically released by the inducible enzyme, inducible NO synthase (iNOS). Moreover, it is of paramount importance to note that peroxynitrite, formed by a non-enzymatic reaction of O_2^- with NO, is markedly mutagenic and responsible for tumor development (Maeda & Akaike 1998, Yoon *et al* 2008). Collectively, suppression of leukocytic free radical generation may reduce the risks of carcinogenesis. In accordance with this, we are accumulating data that shows that some food factors exhibit pronounced cancer preventive activity in rodent models (Murakami *et al* 2000, Nakamura *et al* 1998, Ohata *et al* 1998), and one of the notable action mechanisms by which they suppress carcinogenesis may be an attenuation of the O_2- and NO generation pathways (Murakami *et al* 2000, Nakamura *et al* 1998, Ohata *et al* 1998).

Cancer chemoprevention is currently regarded as one of the most promising avenues in cancer control (Wattenberg LW 1985, Tanaka T 1992). Several types of phytochemicals, such as carotenoids, flavonoids or anti-oxidative vitamins C and E, have been believed to reduce cancer incidence in humans (Hirayama T 1978). However, knowledge of their biological functions is limited.

A halophyte is a plant that naturally grows where it is affected by salinity in the root area or by salt spray, such as in saline semi-deserts, mangrove swamps, marshes and sloughs, and seashores (Glenn *et al* 1998).

There is a need to discover new types of chemopreventive agents by using bioassay systems. Thus, we are interest in humble plant, such as halophyte.

As a new approach to search for cancer preventive plant factors, we conducted edible halophyte for inhibitory activities toward NO generation in LPS/IFN-γ-stimulated mouse macrophage RAW 264.7 cells.

Materials and Methods

1. Chemicals and Cells

Lipopolysaccharide (LPS) (*E. coli* serotype 0127, B8), trypsin with EDTA 2.25% (1×) and L-arginine were purchased from Difco Labs (Detroit, MI, USA), Hyclone Co. (USA) and Waco Pure Chemicals Co. Ltd (Osaka, Japan), respectively. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS) and interferon (IFN)-γ were purchased from Gibco BRL (Grand Island, NY, USA). Dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) and other chemicals were purchased from Sigma Inc. (St. Louis, MO, USA). RAW 264.7 cells, the mouse machrophage cell line, was obtained from ATCC (USA).

2. Methanolic Extraction and Solvent Fractionation

Artemisia fukudo Makino (AF) were directly harvested in the middle of June, 2007 from damp ground around saltpan in Jeung-do, Jeonnam, Korea. The fresh plant was washed, dried to remove moisture quickly, and extracted with 100% methanol for 24 h at room temperature three times. Also the fresh plant was extracted with 80℃ hot distilled water for 24 h. The combined methanolic extract and hot water extracts were filtered and dried on a rotary evaporator at temperature below 40℃, and the resulting product was designated as MeOH extracts(AFM) and hot water extracts (AFHW). For the purpose of fractionation, MeOH extract dissolved in distilled water was fractionated by successive solvent extraction with chloroform ($CHCl_3$), hexane (hexane), ethylacetate (EtOAc) and *n*-butanol (BuOH) (Fig. 1). Each fraction was evaporated to dryness under vacuum, and stored at −80℃, and redissolved in DMSO prior to use. All reagents were purchased from Sigma (USA), unless otherwise stated.

3. Cell Culture

RAW 264.7 cells were cultured in plastic dishes containing Dulbecco's modified eagle medium (DMEM) supplemented

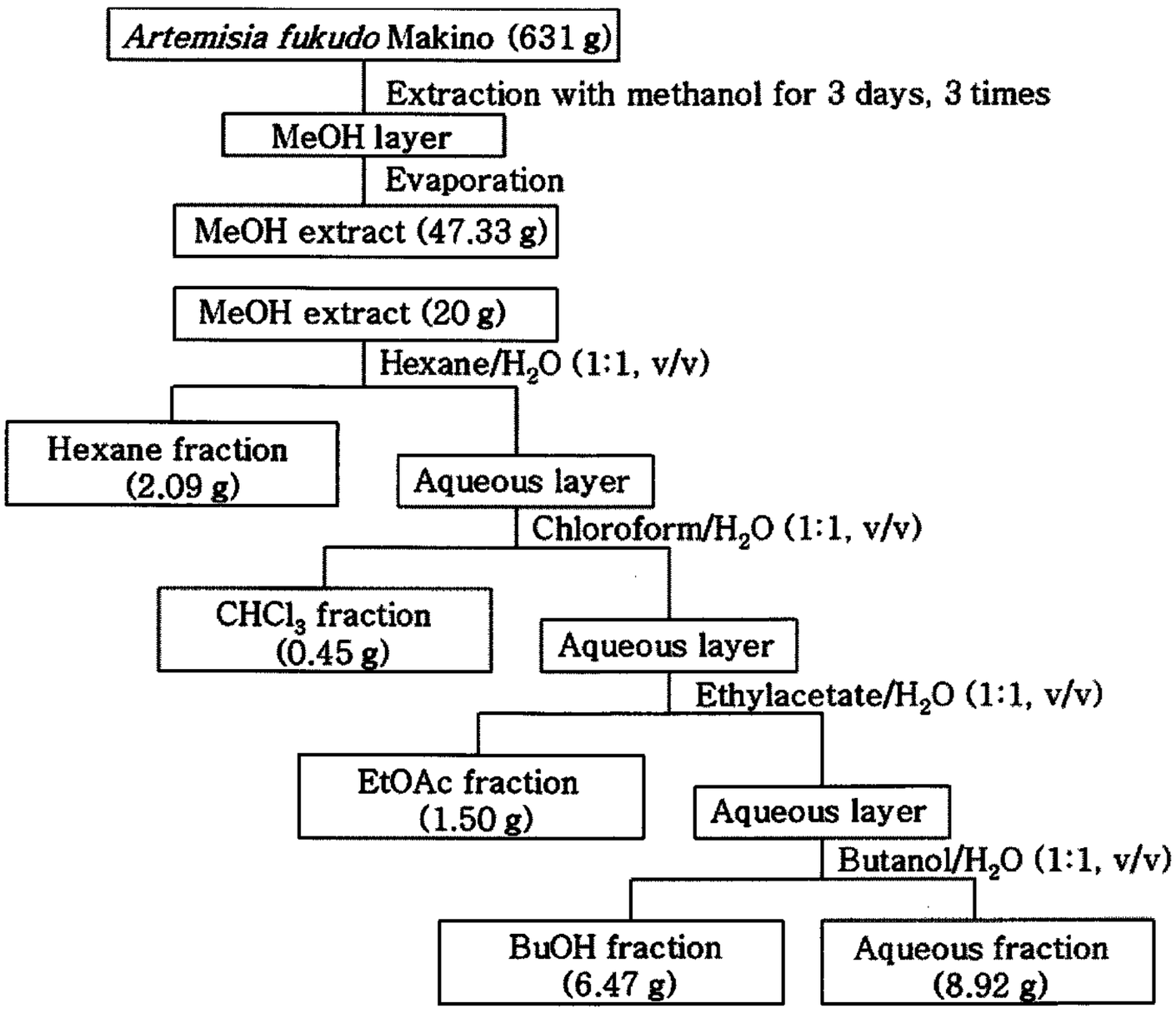

Fig. 1. The procedure for the solvent fractionation of methanol extract from the fresh whole plant of *Artemisia fukudo* Makino.

with 10% FBS in a CO_2 incubator (5% CO_2 in air) at 37℃ and subculture every three days at a dilution of 1:5 using 0.05% trypsin-0.02% EDTA in phosphate-buffered saline (PBS). The cells were cultured in 24-well plate (5×10^5) containing DMEM supplemented with 10% FBS for a day to become nearly confluent. Then cells were cultured with vehicle or *Artemisia fukudo* Makino MeOH extract and its fractions in the presence of concentrations ranging from 1~100 μg/mL dissolved in dimethylsulfoxide (DMSO) and incubated for 48 h [final DMSO concentration 0.025% (v/v)].

4. LPS/IFN-γ-induced NO Generation Test

A mouse macrophage cell line, RAW 264.7, was cultivated in DMEM medium containing L-glutamine supplemented with 10% heat-inactivated (55℃, 30 min) FBS, 200U/mL penicillin and 250 μg/mL streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 (Tayeh & Marletta 1989). The cells were suspended in DMEM medium at a density of 2×10^5 cells/mL, and then treated with LPS (100 ng/mL) and IFN-γ (100 U/mL), L-arginine (2 mM) and 10~100 μg/mL of test extracts. After 24 h, the level of nitrite (NO_2^-) in each test was measured to determine the total NO generation-inhibitory rate (IR) relative to the generation in a control experiment without any test compound as described below. Cells treated with the sample and vehicle without stimulation, and with LPS/IFN- were used as test sample, controls and positive controls, respectively.

5. Cell Viability

Mitochondrial respiration, an indicator of cell viability, was measured by a mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazoliumbromide (MTT) assay. Cells in 24-well plates were incubated with MTT (0.25 mg/mL) for 4 h. The cells were solubilized in 0.04 N HCl in isopropanol. The extent of the reduction of thiazolyl blue tetrazolium bromide (MTT) within cells was quantitated by the measurement of absorbance at 570 nm (Sladowski *et al* 1992).

6. Measurement of NO_2^- Formation

NO_2^- production, an indicator of NO synthesis, was determined in the supernatant of the media by the Griess reaction as reported previously (Greenwald et al 1995). After the 24 h incubation of mouse macrophage cells, the supernatants (0.5 mL) were added to a solution of 0.5 mL of the Griess reagent (1% sulfanilamide, 0.1% naphythylethylene diamine dihydrochloride in 5% H_3PO_4) to form a purple azodye with an absorption maximum at 543 nm.

7. Measurement of L-Citrulline

The L-citrulline level in the medium was determined colorimetrically by reaction of the supernatant of the medium with diacetyl monoxime as reported previously (Boyde & Rahmatullah 1980). Briefly, 0.6 mL of a chromogenic reagent (5 mg thiosemicarbazide in reagent 1/reagent 2, 2:1) was added to 0.4 mL supernatant. The reaction mixture was heated at 100℃ for 5 min followed by measurement of the visible absorption at 530 nm. Reagent 1 was composed of 250 mL concentrated sulfuric acid, 200 mL concentrated phosphoric acid and 550 mL distilled water. After the mixture was cooled down to room temperature, $FeCl_3$ (250 mg) was added to the solution. Reagent 2 was made by resolving to 500 mg of diacetyl monoxime in 100 mL distilled water.

8. Statistical Analysis and Inhibitory Rate (IR)

Each experiment was performed at least three times, and the inhibitory rates against NO generation are shown as mean with standard deviation (mean±SD) values. The statistical significance of differences between groups in each assay was assessed by a Student's *t*-test (two sided) that assumed unequal variance. The IR in each assay was calculated by the following equation:

$$IR\ (\%) = \{1-[(\text{Test sample data})-(\text{Negative control data})] \times [(\text{Positive control data})-(\text{Negative control data})]^{-1}\} \times 100$$

Results and Discussion

1. Effects of AFM and AFHW on NO Generation

Macrophage inducible nitric oxide synthase is able to generate massive amounts of NO which contributes to the host immune defense against viruses and bacteria. Monocyte-macrophages stimulated with the bacterial wall component LPS and cytokines such as IFN-γ express the inducible form of nitric oxide synthase (iNOS).

Fresh *Artemisia fukudo* Makino was extracted with methanol at room temperature. And then solvent fractionation of methanol extract was done with *n*-hexane, chloroform, ethyl acetate and buthanol, respectively (Fig. 1). The yields of the extract and fractions were as follows (w/w): MeOH extract; 9.47%, hexane fraction; 10.44%, $CHCl_3$ fraction; 2.23%, EtOAc fraction; 7.50%, BuOH fraction; 32.47%, aqueous (H_2O) fraction; 44.62% (Fig. 1). The effects of methanol and hot water extracts of *Artemisia fukudo* Makino (AFM and AFHW, respectively) on nitric oxide (NO) generation was investigated in LPS- and IFN-γ-stimulated RAW 264.7 cells.

Artemisia fukudo Makino is one of halophyte, compositae family, that growing on marshy areas of around saltpan was capable of being used as food in a famine.

Methanol extracts and hot water extracts of fresh AF were tested for NO generation inhibitory activities in a mouse macrophage cell line, RAW 264.7 which was stimulated with both LPS (100 ng/mL) and IFN-γ (100 U/mL). AFM displayed a stronger inhibited iNOS activity and nitrite generation activity than AFHW with significant cell viability (CV≧80%) (Fig. 2).

2. Inhibition Effects of AF Fractionations on Total NO Generation

The total activity showing the level of nitrite production was measured by the method of Griess (Green *et al* 1982). There are at least two pathways to show the iNOS inhibitory activity: (1) inhibition of signal transduction for the iNOS gene expression (protein kinase C, tyrosine kinase, mitogen-activated protein kinase, activating protein-1, nuclear factor-κB, etc.) (Lowenstein *et al* 1993, Nakayama *et al* 1994, Spink *et al* 1995); (2) direct inhibition of iNOS enzyme activity (Spink *et al* 1995). iNOS inhibitory activity resulting from these two pathways was measured by monitoring the level of L-citrulline selectively produced from L-arginine by the action of iNOS (Szabo *et al* 1993, Szabo *et al* 1994).

When $CHCl_3$ fraction samples exhibited marked cytotoxicity (cell viability<50%) at 20 and 100 μg/mL, the concentration was decreased to 10 μg/mL. No cytotoxicity was observed at this concentration (CV≧60%), and AF$CHCl_3$ remarkably inhibited total NO and iNOS activity at 10 μg/mL. The triplicate experiments of each sample at 10, 20 and 100 μg/mL were carried out to give the mean±SD (Fig. 6). Inhibitory activities of the extracts for the total activity, iNOS inhibitory activity and cell viability are shown in Fig. 3~6. The cell viability of each extract was measured by MTT assay and that of the positive control (LPS+IFN-γ) was standardized as 100%.

As for the total inhibition, Hexane and MeOH fractions showed strongly inhibitory active (IR=92.1 and 90.7%, respectively) at 100 μg/mL, and $CHCl_3$ fractions showed strongly inhibitory active (IR=67.8%) at 10 μg/mL, with significant cell viability(CV≧60%). EtOAc fractions showed strong inhibitory active (IR=53.1%) for total NO generation at 10 μg/mL, with significant cell viability(CV≧60%) (Fig. 3 and Fig. 6).

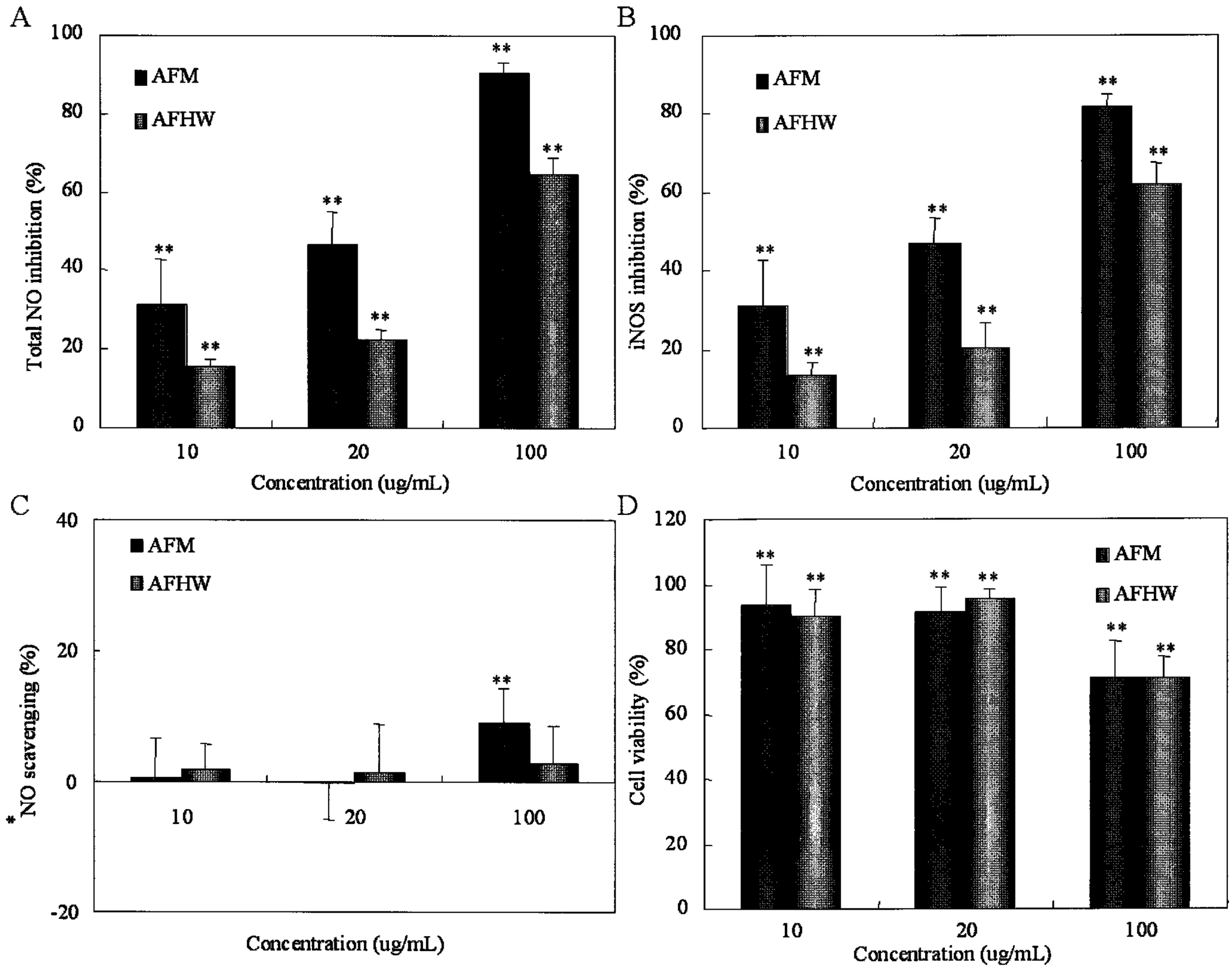

Fig. 2. Inhibitiory effects of total and iNOS activities for methanol extracts and hot water extracts from *Artemisia fukudo* Makino (AFM and AFHW, respectively) in LPS- and interferon-γ-stimulated RAW 264.7 cells.
Data shown are mean±SD., A; Total NO inhibition(%), B; iNOS inhibition(%), C; NO scavenging(%), *NO scavenging=total inhibition(%)− iNOS inhibition(%), D; Cell viability(%). ** $p<0.01$ vs. control.

BuOH fractions and water fractions showed inactive for inhibition of total NO generation.

3. Inhibition Effects of AF Fractionations on Total iNOS Activity

The tendency in the inhibitory proportion of the iNOS inhibitory activity at 100, 20 and 10 μg/mL was quite similar to that in the total inhibitory activity (Fig. 4).

As for the iNOS inhibitory activity, hexane fractions and MeOH fractions showed strongly inhibitory activity (IR=85.0 and 81.6%, respectively) at 100 μg/mL, hexane fractions showed markedly inhibitory activity (IR=61.2%) at 20 μg/mL, and $CHCl_3$ fractions showed remarkably inhibitory activity (IR=71.2%) even decreased at 10 μg/mL, with significant cell viability(CV≧60%) (Fig. 4 and Fig. 6).

EtOAc fractions showed weakly iNOS inhibitory activity (IR=41.4%) at 10 μg/mL, with significant cell viability(CV≧60%) (Fig. 4 and Fig. 6). BuOH fractions and water fractions showed inactive for inhibition of iNOS activity.

4. Effects of AF Fractionations on NO Scavenging

The remarkable inhibitory rates of AFM, hexane and $CHCl_3$ fractions for total NO generation must be attributed to the inhibition of iNOS activity rather than the NO scavenging effect, even though more detailed analyses should be required (Fig. 5).

The present study demonstrates that the fractionation ex-

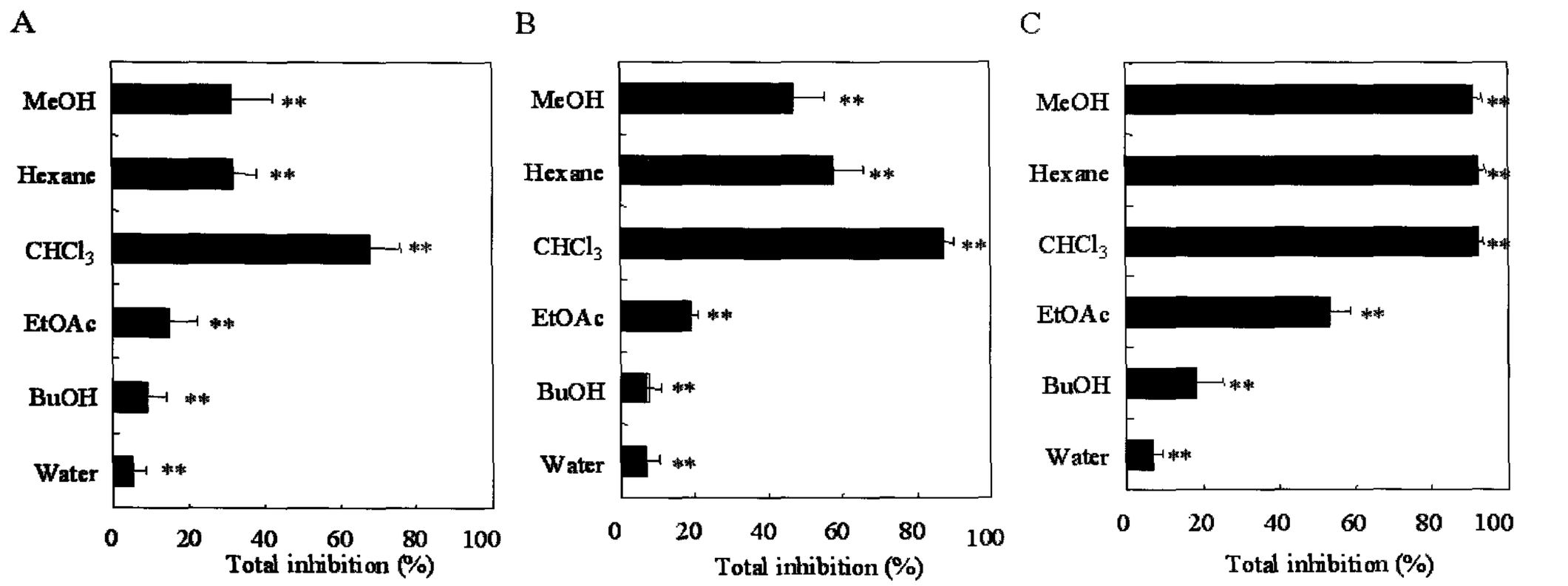

Fig. 3. Inhibitiory effects of total NO generation for fractionation extracts from *Artemisia fukudo* Makino in LPS- and interferon-γ-stimulated RAW 264.7 cells.

Data shown are mean±SD., A; at 10 μg/mL concentration, B; at 20 μg/ mL concentration, C; at 100 μg/mL concentration. MeOH; methanol extracts, Hexane; hexane fraction extracts, EtOAc; ethyl acetate fraction extracts, $CHCl_3$; chloroform fraction extracts, BuOH; buthanol fraction extracts, Water; aqueous fraction extracts. $^{**}p<0.01$ vs. control.

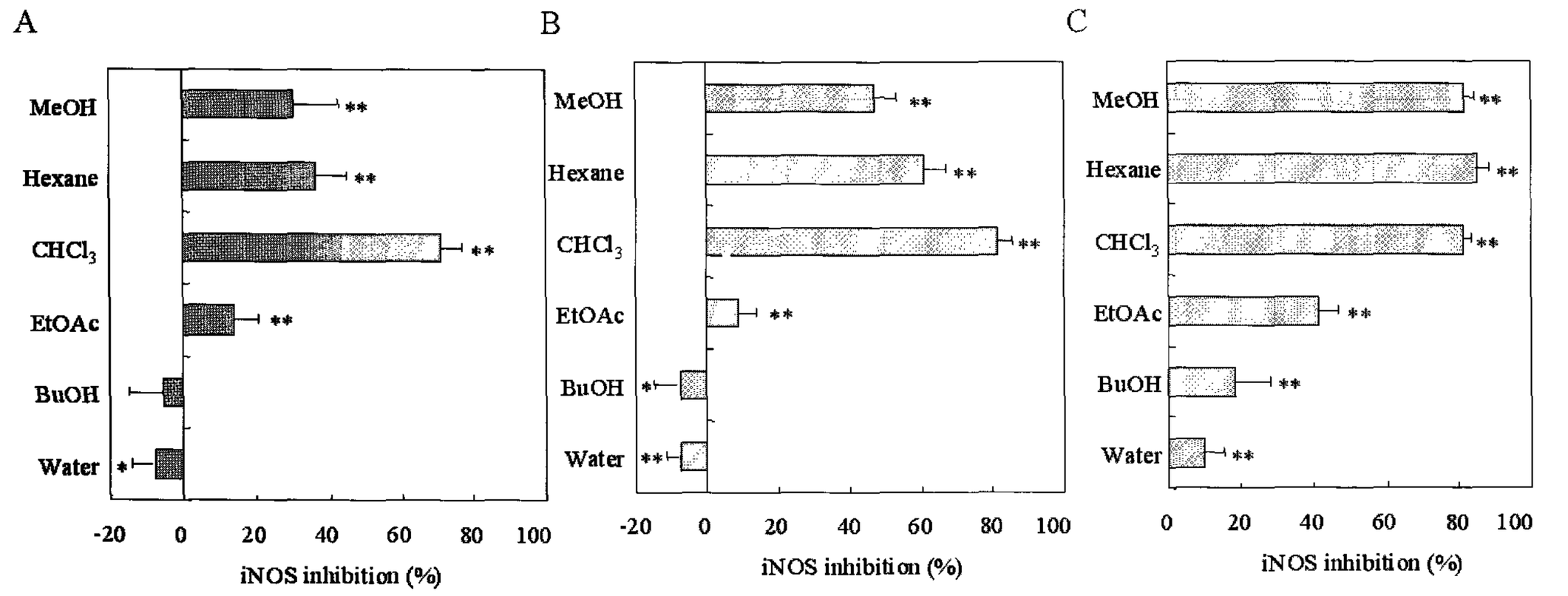

Fig. 4. Inhibitory effects of iNOS generation for fractionation extracts from *Artemisia fukudo* Makino in LPS- and interferon-γ - stimulated RAW 264.7 cells.

Data shown are mean±SD., A; at 10 μg/mL concentration, B; at 20 μg/mL concentration, C; at 100 μg/mL concentration. MeOH; methanol extracts, Hexane; hexane fraction extracts, EtOAc; ethyl acetate fraction extracts, $CHCl_3$; chloroform fraction extracts, BuOH; buthanol fraction extracts, Water; aqueous fraction extracts. $^{*}p<0.05$; $^{**}p<0.01$ vs. control. Methanol extracts and hot water extracts from *Artemisia fukudo* Makino(AFM and AFHW, respectively).

tracts from AF of edible halophyte have inhibitory effects on LPS/IFN-γ-stimulated NO generation in RAW 264.7 cells. It is necessary to test the influence of iNOS-inhibitable extracts on cNOS activity, which is known to regulate blood pressure. It is still unclear whether the inhibitory effects of edible halophyte on NO generation even partly reflect beneficial aspects of edible halophyte ingestion for cancer prevention.

It is important to note that the AF(compositiae) exhibited strong total inhibitory activity (IR≧90%). In particular, the total activities of hexane fraction samples at 100 μg/mL were markedly high (IR=92.1%). Even when the sample concentration was decreased to 10 μg/mL, $CHCl_3$ fractions exhibited considerable cytotoxicity and showed strong total activity (IR ≧60%) with high viability (CV≧60%) (Fig. 3 and Fig. 6).

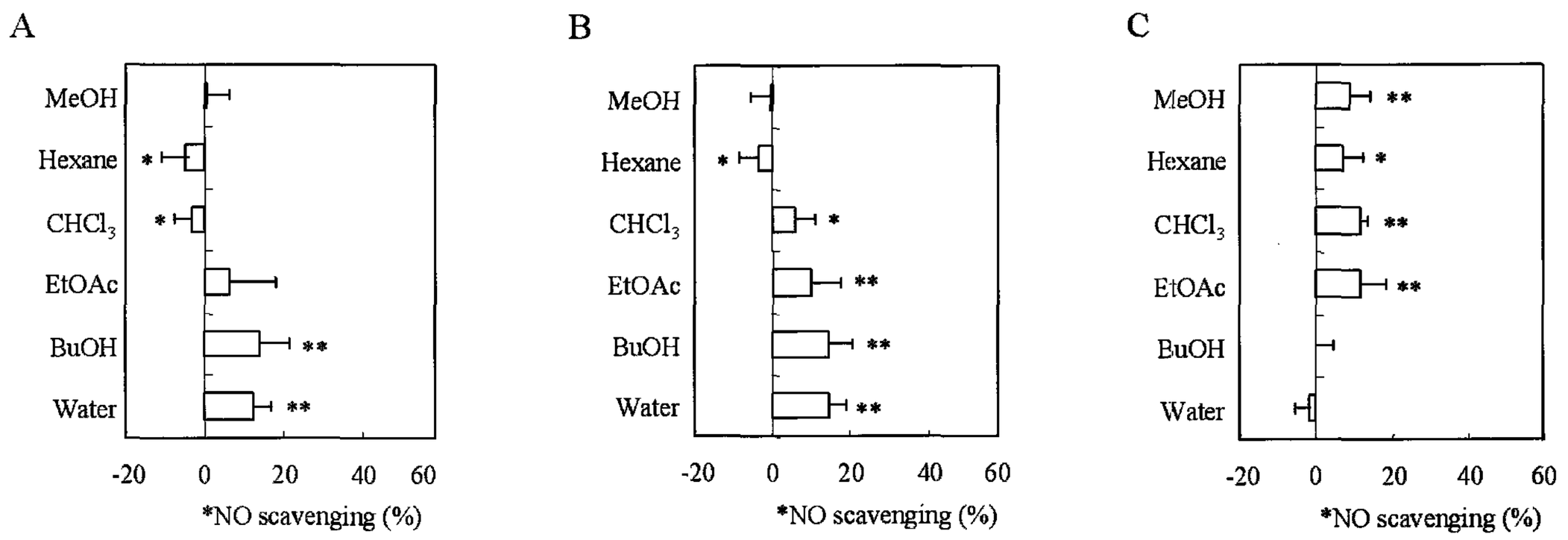

Fig. 5. NO scavenging effects of fractionation extracts from *Artemisia fukudo* Makino in LPS- and interferon-γ-stimulated RAW 264.7 cells.

Data shown are mean±SD., *Difference= total inhibition (%)-iNOS inhibition (%). A; at 10 μg/mL concentration, B; at 20 μg/mL concentration, C; at 100 μg /mL concentration. MeOH; methanol extracts, Hexane; hexane fraction extracts, EtOAc; ethyl acetate fraction extracts, $CHCl_3$; chloroform fraction extracts, BuOH; buthanol fraction extracts, Water; aqueous fraction extracts. * $p<0.05$, ** $p<0.01$ vs. control.

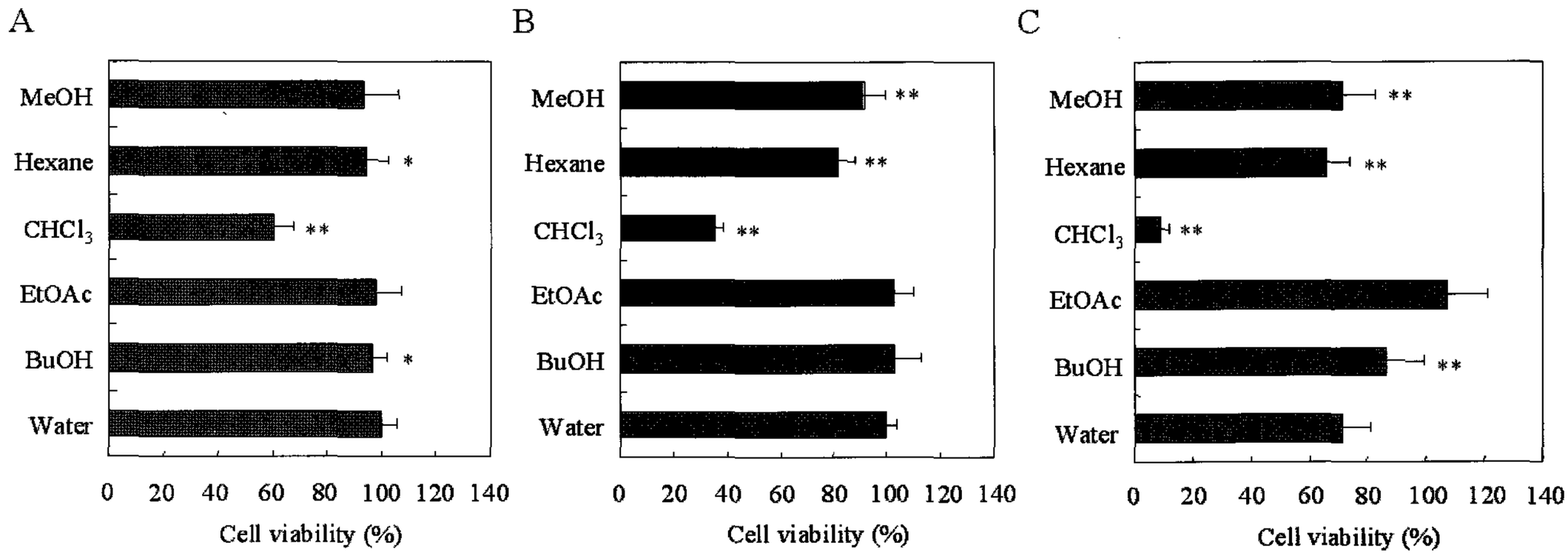

Fig. 6. Cell viability of fractionation extracts from *Artemisia fukudo* Makino in LPS- and interferon-γ-stimulated RAW 264.7 cells.

Data shown are mean±SD., A; at 10 μg/mL concentration, B; at 20 μg/mL concentration, C; at 100 μg/mL concentration. MeOH; methanol extracts, Hexane; hexane fraction extracts, EtOAc; ethyl acetate fraction extracts, $CHCl_3$; chloroform fraction extracts, BuOH; buthanol fraction extracts, Water; aqueous fraction extracts. * $p<0.05$, ** $p<0.01$ vs. control.

Hexane fraction exhibited strong total activity with the strong iNOS inhibitory activity (IR=85.0% and 61.2%, at 100 and 20 μg/mL, respectively) (Fig. 3 and Fig. 4).

A number of compounds inhibiting carcinogenesis through iNOS inhibition are limited at present. In this context, it is important to note that the ω-3 PUFAs may be significant chemopreventors, possibly by suppression of iNOS gene expression (Ohata *et al* 1997). Takahashi *et al* (1997) reported that the remarkable levels of iNOS and cNOS proteins were detected in rat colon treated with carcinogenic azoxymethane. Therefore, reduction of the excess NO level by phytochemicals from edible halophyte may be one of the new and effective strategies for cancer prevention.

In conclusion, we present basic data of new types of effective chemopreventers which may reduce excess NO in biological systems. Further study is required to investigate that isolation and identification of active principles as well as evaluation of their preventive potentials *in vivo*.

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