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Antioxidant effects of *Origanum majorana* L. on superoxide anion radicals

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Abstract

A purified compound with antioxidant properties (28 mg), T3b, was isolated from a methanol extract (10 g) of *Origanum majorana* L. by sequential procedures, with silica gel column, thin-layer, and LH-20 Sephadex gel column chromatographies. The in vitro scavenging activity of T3b on superoxide anion radical ($O_2^{\cdot-}$) was investigated and compared to those of seven commercially available synthetic or natural antioxidants. Of those, the strongest scavenging action was observed in T3b with an IC_{50} of 1.44 μg/ml. The T3b also exhibited significant inhibitory effects on 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced $O_2^{\cdot-}$ generation and hydrogen peroxide formation in differentiated HL-60 cells, indicating that the isolated compound is a potent chemopreventer. The purified compound from *O. majorana* L. was shown to possess both $O_2^{\cdot-}$ scavenging activity and an inhibitory effect against TPA-induced $O_2^{\cdot-}$ generation.

Keywords: *Origanum majorana* L.; Superoxide anion radical; Radical scavenger; Chemopreventer

1. Introduction

Origanum majorana L. (marjoram) is a herbaceous and perennial plant native to southern Europe and the Mediterranean. For food uses, marjoram is employed to flavour sausages, meats, salads and soups (Novak, Christina, Langbehn, Pank, Skoula, Gotsiou, & Franz, 2000). Traditionally, it is used as a folk remedy against asthma, indigestion, headache and rheumatism. However, little is known about the biologically active compounds of marjoram as a medicinal plant, except for its essential oil.

Reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and nitric oxide (NO) are physiological metabolites. Small amounts of ROS are continuously formed during life as a result of the metabolism of oxygen (Fridovich, 1978). Much evidence indicates that exposure to ROS leads to deleterious changes of cell function by a number of alterations, such as lipid peroxidation (Buettner, 1993), enzyme inactivation (Mak, Misra, & Weglicki, 1983), and oxidative DNA damage (Halliwell, 1999). Also, the accumulation of ROS has been postulated to be implicated in the aging process (Beckman & Ames, 1998). Antioxidants are substances that delay or prevent the oxidation of cellular oxidizable substrates. They exert their effects by scavenging ROS, activating a battery of detoxifying proteins, or preventing the generation of ROS. The $O_2^{\cdot-}$ radical is the initiator of a ROS generation system in mitochondria by oxygen reduction. Dismutation of $O_2^{\cdot-}$ is the main mitochondrial source of H_2O_2, which is subsequently reduced to water by catalase or otherwise decomposed by glutathione peroxidase. Therefore, it is effective to scavenge $O_2^{\cdot-}$ for efficient antioxidant defence systems in mitochondria. In a preliminary study, an extract of marjoram displayed an appreciable scavenging effect on $O_2^{\cdot-}$, which

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prompted us to further investigate the compound of the plant.

The free radical scavengers can protect cells from oxidative stress when they are administered prior to (or) concomitantly with a tumor promoter, in terms of the increased detoxification of free radical formation and thus the prevention of many pathophysiological processes (Mates & Sanchez-Jimenez, 2000). For instance, curcumin, a yellow pigment found in tropical gingers, has been reported as both an oxygen-derived radical scavenger and a generation inhibitor of 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced oxidative stress (Nakamura, Ohto, Murakami, Osawa, & Ohigashi, 1998).

In the present study, we isolated the $O_2^{\bullet-}$ scavenger from marjoram and investigated its scavenging ability in comparison with commercially available antioxidants. Furthermore, we examined the inhibitory effect of this compound on TPA-induced $O_2^{\bullet-}$ generation using a differentiated HL-60 cell system.

2. Materials and methods

2.1. Sample and chemicals

Origanum majorana L. (marjoram) was obtained from Hyangwon Spice Co. (Seoul, Korea), and was authenticated by Dr. Sang-In Shim at Seed Bank for Wild Herbaceous Plant Species, Korea University. The voucher specimen was deposited at the same institute. Diethylenetriaminepentaacetic (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), β-nicitinamide adenine dinucleotide (reduced form, NADH), xanthine (XA), hypoxanthine (HPX), and xanthine oxidase (XOD) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactate dehydrogenase (LDH) was from Boehringer Mannheim (Mannheim, Germany). The human promyelocytic leukemia cell line, HL-60, was obtained from American Type Culture Collection (Rockville, MD). Roswell Park Memorial Institutes (RPMI) 1640 medium was the product of GIBCO (Grand Island, NY). Fetal bovine serum (FBS) and 12-*O*-tetradecanolyphorbol-13-acetate (TPA) were from Sigma Chemical Co. 2′,7′-Dichlorofluorescein diacetate (DCFH–DA) was purchased from Molecular Probes, Inc. (Leiden, Holland). All other chemicals were of analytical grade.

2.2. Extraction and isolation

The plant material was homogenized in an Ultra Turrax homogenizer (7000 rpm, 20 min). After centrifuging the homogenate (5000× ***g***, 30 min), the supernatant was dried with an evaporator under reduced pressure. The crude extract was then suspended in water and re-extracted with hexane, acetone, methanol and water to give the respective fractions after evaporation. The methanol soluble fraction was applied to a column of silica gel and elution was started with chloroform containing increasing amounts of methanol. Fractions eluted with chloroform-methanol (90:10) were developed on a preparative thin layer chromatogram with a mixture of chloroform and methanol (9:1) as mobile phase. The spot corresponding to a R_f value of 0.31 was further purified by column chromatography on Sephadex LH-20 with 50% methanol as an eluent, yielding two subfractions.

2.3. HPLC analysis

The HPLC was performed using a Young-Lin M-930 instrument equipped with an M-720 UV detector and a reversed-phase C-18 column (Lichrospher, 150-4, Waters). A combination of acetonitrile, methanol, and water (1:3:96) was used as mobile phase with a flow rate of 0.5 ml/min. The UV-absorbing substances were detected at 290 nm.

2.4. Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity

The $O_2^{\bullet-}$ scavenging activities of the crude extracts and compounds were determined by an LDH-NADH oxidation system according to Chan and Bielski (1974) with some modifications. 50 μl of 1 M sodium phosphate buffer (pH 7.0), 250 μl of 0.1 mM XA, 150 μl of 1 mM NADH, 30 μl of LDH, 100 μl of 0.5 mM EDTA, and 50 μl of sample, were put into a test tube. The mixture was incubated for 30 s at 25 °C, and then 10 μl of XOD were added to generate $O_2^{\bullet-}$. The change in absorbance was detected at 340 nm for 1 min. The scavenging activity was calculated as follows;

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\Delta\text{Abs of sample}}{\Delta\text{Abs of blank}}\right) \times 100$$

The $O_2^{\bullet-}$ scavenging activity of the purified compound was further assessed by electron spin resonance (ESR) spectrometry. Briefly, the reaction mixture, which was obtained by mixing 40 μl of 5 mM HPX, 20 μl of 9 M DETAPAC, 20 μl of DMPO, 140 μl of 50 mM phosphate-buffered saline (PBS, pH 7.6) and 20 μl of sample was transferred to a quartz analyzing cell, and placed into the cavity of an electron spin resonance (ESR) spectrometer (ER-200D, Bruker, Rheinstetten, Germany). One minute after the addition of XOD, which generate $O_2^{\bullet-}$, the intensity of the signal of DMPO-OOH spin adduct was measured. ESR spectra were recorded at 37 °C with a centre field 3475 G, modulating frequency 100 kHz, modulation amplitude 1.25 G,

microwave power 2 mW, scan rate 2 G/S, and receiver gain 8×10^5.

2.5. Assay for inhibition of TPA-induced superoxide anion radical generation

The human promyelocytic leukemia cell line, HL-60, was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Stock cultures were maintained in T-25 flasks at 37 °C under a humidified atmosphere of 95% air and 5% CO_2. For cell differentiation, HL-60 cells were treated with 1.25% dimethylsulfoxide (DMSO) for 4 days, under the same atmosphere, to stimulate their differentiation into the granulocyte-like cells. Cells were then harvested, washed with PBS, and suspended at a density of 1×10^6 cells/ml. The production of $O_2^{\bullet-}$ was measured according the method of Murakami, Ohura, Nakamura, Koshimizu, and Ohigashi (1996) with some modifications. Prior to 5 μl of a TPA solution (20 μM) being added, cells were pre-incubated with the sample for 15 min at 37 °C and washed twice with PBS to remove the extracellular test sample. Then, 50 μl of cytochrome *c* (20 mg/ml) were added and the whole re-incubated for another 15 min. After the addition of 5 μl of superoxide dismutase (SOD) solution (15,000 U/ml), the reaction was stopped. The cell suspension was centrifuged and the absorbance of supernatant was measured at 550 nm. The $O_2^{\bullet-}$ was quantified as follows; the level of $O_2^{\bullet-}$ (nmol/ml) $=47.7\times Abs_{550\ nm}$.

2.6. Assay for hydrogen peroxide formation

Hydrogen peroxide was detected by using DCFH-DA as an intracellular fluorescence probe as described by Bass, Parce, Dechatelet, Szeja, Seeds, and Thomas (1983). HL-60 cells were differentiated as mentioned above, and then, 5 μl of DCFH-DA solution (200 μM) were added to the differentiated cell suspension, followed by the incubation for 15 min at 37 °C. After incubation, the sample dissolved in 5 μl of DMSO was added to the cell suspension, and the mixture was re-incubated for another 15 min. Fifteen minutes after 5 μl of a TPA solution (20 μM) were added, the reaction was stopped by the addition of 50 μl of an EDTA solution (10 mM). The cell suspension was centrifuged and cells were washed with PBS. The fluorescence, formed by the reaction of DCFH with intracellular hydroperoxides, was measured by a Beckton Dickinson FACS Calibur™ flow cytometer (San Jose, CA).

2.7. Statistical analysis

Data were analyzed by the difference between means, and statistical significance was calculated from Fisher's least significant difference (LSD) or Student's *t*-test.

3. Results and discussion

3.1. Isolation of superoxide anion radical scavenger from Origanum majorana L.

The methanol extractives (10 g) of *O. majorana* L. (marjoram), which exhibited strong $O_2^{\bullet-}$ scavenging activity (85.5%), assayed by an LDH-NADH oxidation system, were divided into water-, chloroform-, ethyl acetate-, and butanol-solubles. Of those, the ethyl acetate fraction showed the highest scavenging activity (data not shown), which led to the further fractionation by sequential procedures with silica gel column, thin-layer and Sephadex LH-20 gel column chromatographies. As shown in Fig. 1, a relatively high scavenging activity was found in T3b (28 mg) between two subfractions obtained by the Sephadex LH-20 gel column chromatography. The purified $O_2^{\bullet-}$scavenging compound, T3b (UV λ_{max} value of 290 nm), showed a single peak on HPLC, indicating that it was highly purified (Fig. 2). The purified compound reacted with Folin–Ciocalteu's reagent, H_2SO_4 and 1 N NaOH, while no reaction was observed with anthrone, Molish, ninhydrin, Ehrlich or ferric chloride reagents. These results implied that $O_2^{\bullet-}$scavenger from marjoram might be a phenolic compound. The scavenging action of plant constituents has been found to be associated with phenolic compounds (Madsen, Nielsen, Bertelsen, & Skibsted, 1996) as well as caffeic acid derivatives and flavonoids (Namiki, 1990).

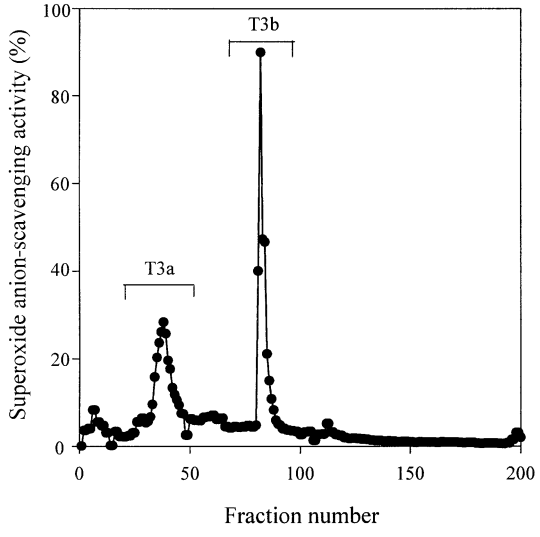

Fig. 1. Purification of antioxidant compound from *Origanum majorana* L. by Sephadex LH-20 gel column chromatography. The scavenging activity (●) of each fraction on superoxide anion radicals was determined by an LDH-NADH oxidation system. Fraction size and flow rate of column chromatography, eluted with 50% methanol were 0.5 ml/tube and 3 ml/min.

3.2. In vitro scavenging effect on superoxide anion radical

The use of synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), has been restricted in the food industry due to their carcinogenesis. Subsequently, interest in the development of antioxidants with limited cytotoxicity is increasing. Ascorbic acid, α-tocopherol, quercetin, epigallocatechin gallate (EGCG), and epicatechin have been known as the effective antioxidants of natural origin (Frankel, 1996; Rajalakshimi & Narasimhan, 1996). Table 1 compares the $O_2^{\bullet-}$scavenging activity of T3b, the purified compound from marjoram, to the commercially available antioxidants under an LDH-NADH oxidation system. The order of IC_{50} of these compounds, i.e. the concentration required for 50% inhibition of NADH oxidation, was found to be: Tb3 (1.44 μg/ml) > EGCG (2.07 μg/ml) > quercetin (3.19 μg/ml) > epicatechin (3.24 μg/ml) > BHA (5.73 μg/ml) > α-tocopherol (6.66 μg/ml) > ascorbic acid (8.05 μg/ml) > BHT (40.8 μg/ml). T3b therefore exhibited the strongest $O_2^{\bullet-}$ scavenging ability. Its activity was approximately five times greater than that of α-tocopherol, which is a natural $O_2^{\bullet-}$ scavenger with high efficiency (Lass & Sohal, 2000). These results suggest that the compound isolated from marjoram should receive attention as a new naturally occurring antioxidant.

The antioxidative properties of T3b were further studied by ESR spectrometry. The ESR spin trapping technique allows the accurate measurement of radical-scavenging activity by an indirect and specific detection of free radical (Madsen, Nielsen, Bertelsen, & Skibsted, 1996). When DMPO was added to a solution of the HPX-XOD reaction system, the spin adduct DMPO-OOH, was formed. As shown in Fig. 3, the signal intensities of DMPO-OOH markedly decreased with increased amounts of T3b added (3.3 ng, 67 ng and 670 ng) to the system, indicating that it contained appreciable $O_2^{\bullet-}$ scavenging activity in a dose-dependent manner. It was reported that the antioxidant compound isolated from young green barley leaves showed a dose-dependent inhibitory activity towards superoxide generation and the phenolic moiety of the structure was

Table 1
Scavenging activities of antioxidant compound from *Origanum majorana* L. and commercial antioxidants on superoxide anion radicals[a]

Antioxidant[b]	IC_{50}[c] on superoxide anion radical (μg/ml)
BHT	5.73±0.21 c[d]
BHA	40.80±2.35 a
Ascorbic acid	8.05±1.18 b
α-Tocopherol	6.66±1.07 bc
EGCG	2.67±0.29 d
Epicatechin	3.24±1.01 d
Quercetin	3.19±0.84 d
T3b	1.44±0.16 e

[a] Data represent the mean±S.D. of three replicates.
[b] BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; EGCG, epigallocatechin gallate; and T3b, antioxidant compound from *origanum majorana* L.
[c] IC_{50} = the concentration required for 50% inhibition of NADH oxidation
[d] Values in a column indicated with different letters are significantly different ($P = 0.05$).

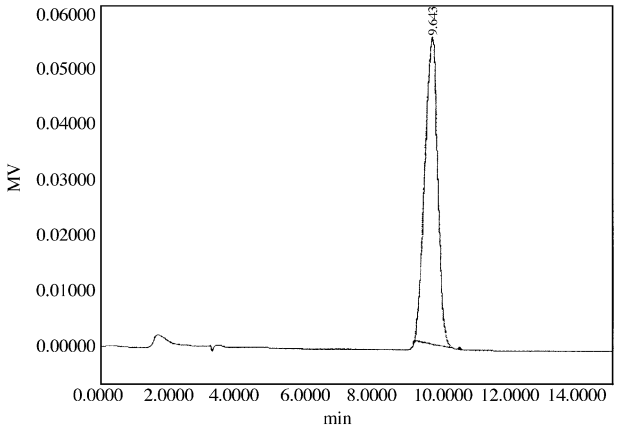

Fig. 2. HPLC profile of antioxidant compound from *Origanum majorana* L. The HPLC analysis was performed on a Young-Lin M-930 instrument, using a reversed-phase C-18 column (3.9×150 mm) column and an M-720 UV detector at 290 nm. A mixture of acetonitrile, methanol and water (1:3:96, v/v/v) was used as mobile phase with a flow rate of 0.5 ml/min.

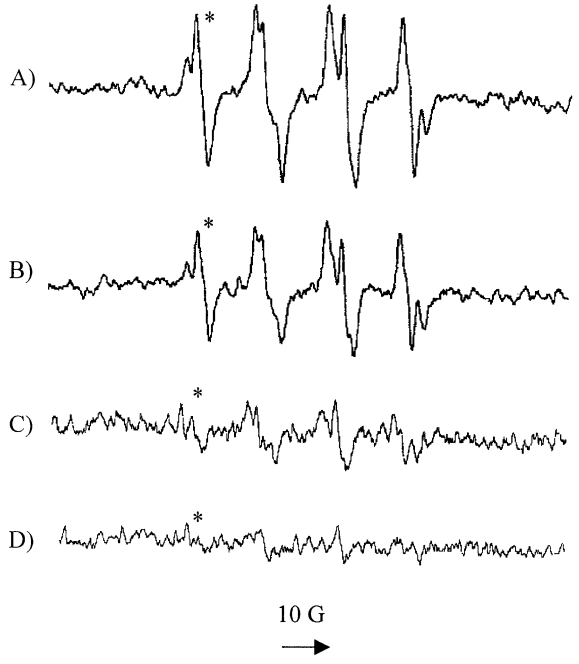

Fig. 3. Electron spin resonance (ESR) spectra effects of antioxidant compound from *Origanum majorana* L. on the formation of spin adduct of superoxide anion radical (DMPO–OOH) generated from the hypoxanthine-xanthine oxidase system. A; control, B; in the presence of 3.3 ng of sample, C; in the presence of 67.0 ng of sample, and D; in the presence of 670 ng of sample. * DMPO-OOH.

presumably responsible for its ability to scavenge free radicals (Arimoto, Ichinose, Yoshikawa, & Shibamoto, 2000). Based upon these results, we could confirm that the inhibitory mechanism of T3b is due to scavenging of $O_2^{\bullet-}$ by the action of SOD, which destroys the $O_2^{\bullet-}$ by converting it to H_2O_2 that can in turn be destroyed by catalase or glutathione peroxidase reactions. The proposed mechanism of T3b under the ESR spectrometry is Eq. (1) the production of $O_2^{\bullet-}$ in HPX-XOD reaction, is reduced by the scavenging activity of T3b, and, subsequently [Eq. (2)] the spin adduct formation by reaction between radical and spin trap DMPO is decreased.

$$O_2^{\bullet-} + 2AH \rightarrow H_2O_2 + 2A\cdot \quad (1)$$

$$O_2^{\bullet-} + DMPO \rightarrow DMPO\text{-}OOH \downarrow \quad (2)$$

3.3. In vitro inhibitory effects on TPA-induced superoxide anion radical generation and intracellular hydrogen peroxide formation

Fig. 4 shows the inhibitory activities of purified compounds from marjoram and genistein, a well-known antitumor promoter, isolated from soybean, on TPA-induced $O_2^{\bullet-}$ generation in differentiated HL-60 cells. The $O_2^{\bullet-}$ was generated in granulocyte-like HL-60 cells by stimulation with TPA, and was measured by reduction of cytochrome *c*. The T3b inhibited cytochrome *c* reduction by 78% at the concentration of 50 μg/ml after stimulation with TPA. On the other hand, the inhibitory activity of genistein was slightly lower than that of T3b. The IC_{50} values of T3b and genistein in a TPA-induced $O_2^{\bullet-}$ generation system in vitro were 17.3 μg/ml and 24.7 μg/ml, implying that the compound from marjoram was a potent antitumor promoter, via inhibiting $O_2^{\bullet-}$ generation. A close relationship between the inhibition of $O_2^{\bullet-}$ generation and the antitumor promotion has been observed in some natural chemopreventers (Murakami Ohura, Nakamura, Koshimizu, & Ohigashi, 1996). The inhibitory effect of T3b might be, at least in part, due to the inhibition of NADPH oxidase, since it is a key enzyme in the $O_2^{\bullet-}$ generation by leukocytes in tumor promotion (Perchellet, Perchellet, Gali, & Gao, 1995). Note that, in the present investigation, we eliminated the possibility of scavenging effect of T3b by removing the compound before the stimulation with TPA in the $O_2^{\bullet-}$ generation system.

A positive correlation has been reported between $O_2^{\bullet-}$ generation and intracellular hydrogen peroxide formation in granulocytes (Nakamura, Kawamoto, Ohto, Torikai, Murakami, & Ohigashi, 1999). Therefore, the inhibitory effect of T3b against hydrogen peroxide formation in differentiated HL-60 cells, using DCFH-DA as an intracellular fluorescence probe was investigated. As shown in Fig. 5, most of the cells produced hydroperoxides with the TPA stimulation alone (hydroperoxide positive percentage = 71.9%). However, hydroperoxide formation was significantly inhibited

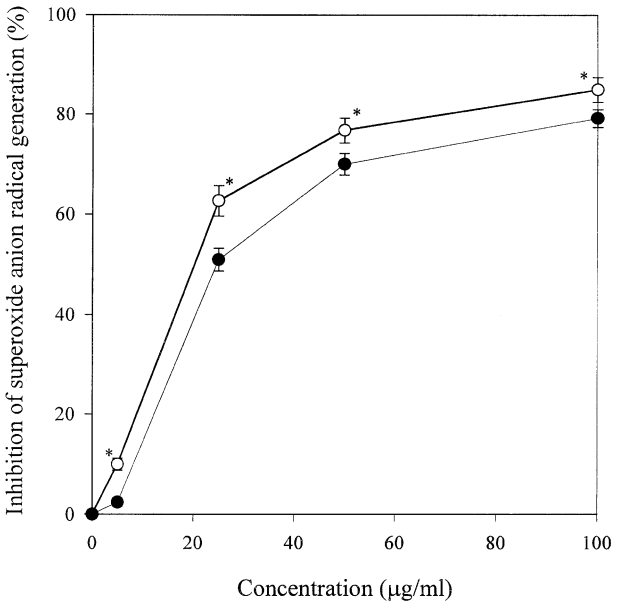

Fig. 4. Inhibitory effects of antioxidant compound from *Origanum majorana* L. and genistein on 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced superoxide anion radical generation in differentiated HL-60 cells. ○; Antioxidant compound from *Origanum majorana* L., and ●; genistein. Statistical analysis was performed using a Student's *t*-test: $*P < 0.05$ versus genistein.

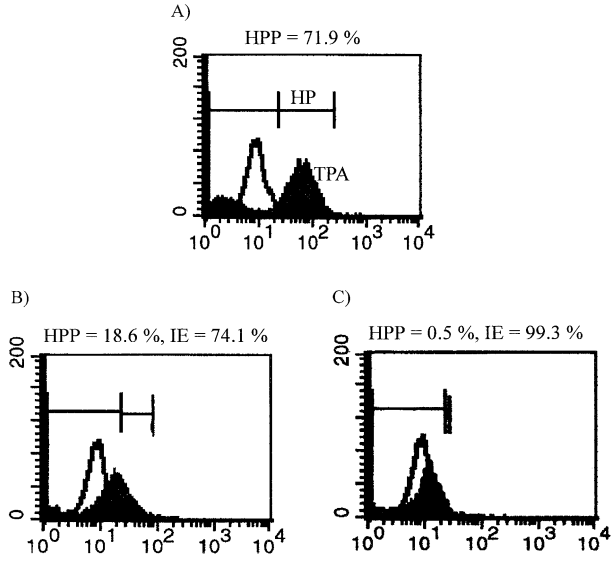

Fig. 5. Fluorescence distribution of antioxidant compound from *Origanum majorana* L. on 12-*O*-tetradecanolyphorbol-13-acetate (TPA)-induced intracellular hydrogen peroxide formation in differentiated HL-60 cells. The DCF fluorescence was monitored on a flow-cytometer with excitation and emission wavelengths at 488 nm and 525 nm, respectively. Control value regarded as hydroperoxide positive (HP), and its percentage was expressed as the hydroperoxide positive percentage (HPP). The IE represents inhibitory effect (%). A, TPA alone; B, TPA + 25 μg of sample; and C, 25 μg of sample. *X*-axis, relative fluorescence intensity; and *y*-axis, cell count.

when T3b was introduced into the assay system. Its inhibitory effects (IE) at concentrations of 25 μg/ml and 50 μg/ml were 74.1 and 99.3%, respectively, indicating the complete suppression of intracellular hydrogen peroxide formation with the treatment of T3b at a concentration of 50 μg/ml. Combined with the results of in vitro inhibitory effect of T3b on TPA-induced $O_2^{\bullet-}$ generation, it appears that that most of the intracellularly-formed hydrogen peroxides are derived from $O_2^{\bullet-}$ generation, which is in general agreement with the observations by Lundqvist, Follin, Khalfan, and Dahlgren (1996).

4. Conclusions

Exposure to $O_2^{\bullet-}$ causes cell injuries or death to the aerobic organisms via cytotoxicity, carcinogenicity or mutagenicity. Removing $O_2^{\bullet-}$ can successfully protect cells against this damage. Therefore, small molecules, with antioxidant properties, are the new tools for the prevention of such diseases. In the present study, the compound from marjoram, T3b, which exhibited antioxidant properties was isolated. A high $O_2^{\bullet-}$ scavenging activity of T3b might exert a beneficial action against pathophysiological alterations caused by the presence of $O_2^{\bullet-}$. Interestingly, T3b also has an inhibitory action against TPA-induced $O_2^{\bullet-}$ generation, and can be described as an effective chemopreventer. These results, confirm that T3b does possess both scavenging action against $O_2^{\bullet-}$ and protective action against TPA-induced tumor promotion. Research is underway to characterize the structural identification of the antioxidant compound from marjoram and to evaluate its in vivo effects as a radical-scavenger and an antitumor-promoter.

Acknowledgements

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