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Ramalin, a novel nontoxic antioxidant compound from the Antarctic lichen *Ramalina terebrata*

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ABSTRACT

Ramalin (γ-glutamyl-N′-(2-hydroxyphenyl)hydrazide), a novel compound, was isolated from the methanol–water extract of the Antarctic lichen *Ramalina terebrata* by several chromatographic methods. The molecular structure of ramalin was determined by spectroscopic analysis. The experimental data showed that ramalin was five times more potent than commercial butylated hydroxyanisole (BHA) in scavenging 1-diphenyl-2-picryl-hydazil (DPPH) free radicals, 27 times more potent in scavenging 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid free radicals ($ABTS^{\bullet+}$) than the vitamin E analogue, trolox, and 2.5 times more potent than BHT in reducing Fe^{3+} to Fe^{2+} ions. Similarly, ramalin was 1.2 times more potent than ascorbic acid in scavenging superoxide radicals and 1.25 times more potent than commercial kojic acid in inhibiting tyrosinase enzyme activity, which ultimately leads to whitening of skin cells. Ramalin showed no or very little cytotoxicity in human keratinocyte and fibroblast cells at its antioxidant concentration. Furthermore, ramalin was assessed to determine its antioxidant activity *in vivo*. One microgram per milliliter ramalin significantly reduced the released nitric oxide (NO) and 0.125 μg/ml ramalin reduced the produced hydrogen peroxide (H_2O_2) in LPS (lipopolysaccharide)-stimulated murine macrophage Raw264.7 cells. Considering all the data together, ramalin can be a strong therapeutic candidate for controlling oxidative stress in cells.

Introduction

Living organisms accumulate reactive oxygen species (ROS) through both normal metabolic processes and exogenous sources. ROS, such as superoxide anions (O_2^-), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and hypocholorous acid (HOCl), are associated with inflammation, cardiovascular diseases, cancer, aging-related disorders, metabolic disorders, and atherosclerosis (Ames et al. 1993). ROS are dangerous because they attack unsaturated fatty acids and cause membrane lipid peroxidation, decreases in membrane fluidity, loss of enzyme receptor activities, and damage to membrane proteins, ultimately leading to cell inactivation (Dean and Davies 1993). Despite the existence of natural defense mechanisms, increasing ROS accumulation over the lifetime of a cell can cause irreversible oxidative damage (Tseng et al. 1997). Thus, antioxidant agents that can slow or prevent the oxidation process by removing free radical intermediates are desired. Several strong synthetic antioxidants have been reported (Shimizu et al. 2001); however, they have proven to be highly toxic (Wichi 1988). For this reason it has become necessary to isolate antioxidants from natural sources for use as supplements to human health. A wide range of natural compounds, including phenolic compounds, nitrogen compounds, and carotenoids have antioxidant properties and may serve as potential candidates for antioxidant resources (Velioglu et al. 1998).

Lichens are the symbiotic association of a fungus (mycobiant) and an alga (photobiant) and/or cyanobacteria, and they resemble non-flowering plants. In lichens, the fungus forms a thallus or lichenized stroma that contains characteristic secondary metabolites (Huneck 1999). Several lichen metabolites of various chemical classes, including the aliphatic acids, depsides and depsidones, dibenzofurans, diterpenes, anthraquinones, naphthoquinones, usnic acids, pulvinic acids, xanthones, and epidithiopiperazinediones have been reported with various biological activities such as cytotoxic, fungicidal, antimicrobial, antioxidant, and anti-inflammatory (Müller 2001). Most known antioxidant activities of lichens are derived from the species of tropical or sub-tropical origin. Fewer studies have been conducted on the antioxidant activities of lichens from Antarctic regions

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(Bhattarai et al., 2008; Paudel et al. 2008). Here we present a novel antioxidant compound isolated from the Antarctic lichen *Ramalina terebrata* along with its various antioxidant activities.

Materials and methods

Specimen collection and identification

Ramalina terebrata Hook and Taylor (Ramalinaceae) was collected by one of us (H.D.B.) from Barton Peninsula (S62°13.3′, W58°47.0′) on King George Island, Antarctica, in January 2008. The identification of the species was performed as described previously (Paudel et al. 2008). The voucher specimen is deposited in the lichen herbarium of Korea Polar Research Institute.

Extraction and isolation of ramalin

A freeze-dried and ground lichen sample (672 g) was extracted three times in mixture of methanol and water (5 l, 80:20, v/v). The resulting crude extract (83 g) was dissolved in 1 l of distilled water and sequentially partitioned three times with 1 l *n*-hexane and $CHCl_3$ to yield n-hexane (12.7 g), $CHCl_3$ (9.1 g), and water-soluble (61.0 g) fractions. Of these fractions, the water soluble aqueous extract was highly active against DPPH free radical scavenging activity (IC_{50} = 9 μg/ml). A portion (5 g) of the water-soluble extract was then subjected to automated mild pressure liquid chromatography (MPLC) using a C_{18}ODS column (15 cm × 3 cm) and a stepwise gradient solvent system of 0%, 20%, 40%, 60%, 80%, and 100% methanol in water. The fraction eluted at 0% methanol (2 g) was very active against DPPH free radical (IC_{50} = 8 μg/ml), and a portion (100 mg) of this fraction was subjected to semi-preparative reverse phase HPLC using a C_{18}ODS column (5 μm particle size, 250 mm × 10 mm). The gradient solvent system was 0% methanol in water (0.1% formic acid) over 10 min, 20% methanol over 20 min, and 100% methanol over 30 min. The flow rate was 2 ml/min. Compounds were detected by UV absorption at 280 nm. The fifth fraction (45 mg; t_R = 18.88 min) was found to be the most active against DPPH free radicals (IC_{50} = 1 μg/ml) and was therefore subjected to further purification by using repeated semi-preparative HPLC using a C_{18}ODS column (250 mm × 10 mm). The gradient solvent system was 10–30% acetonitrile in water (0.1% formic acid) over 50 min with a flow rate of 2 ml/min. Ramalin (**1**, 30 mg) was eluted at 8.26 min and showed strong DPPH free radical activity (IC_{50} = 0.99 μg/ml). The molecular formula of ramalin [1,(-glutamyl-N'-(2-hydroxyphenyl)hydrazide, Fig. 1] was determined as $C_{11}H_{15}N_3O_4$ by analysis of its HRESIMS data [m/z 254.1141 $(M+H)^+$; (0.0 mmu)], indicating six degrees of unsaturation. This formula was supported by ^1H and ^{13}C NMR data (Paudel 2009).

Antioxidant assays (in vitro)

Various *in vitro* antioxidant activities such as DPPH and $ABTS^{\bullet+}$ free radical scavenging capacity (Blois 1958; Rice-Evans and Miller 1994), Fe^{3+} reducing power (Oyaizu 1986), superoxide anion scavenging capacity in riboflavin-nitroblue tetrazolium (NBT)-light system (Beauchamp and Fridovich 1971) and tyrosinase inhibitory assay (Higuchi et al. 1993) were determined by comparing to commercially available standard compounds (Table 1). These experiments were modified at various degrees as described previously (Bhattarai et al., 2008).

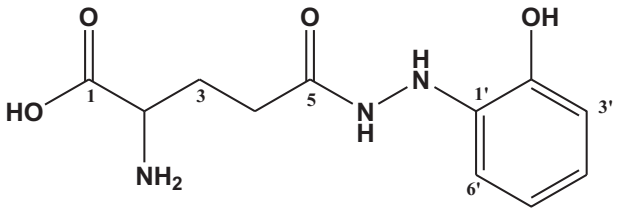

Fig. 1. Antioxidant compound, ramalin isolated from *Ramalina terebrata*.

Antioxidant assays (in vivo)

Nitric oxide and hydrogen peroxide assays were preformed in Murine macrophage Raw264.7 cells as described below.

Cell culture

All reagents were from Sigma (St. Louis, MO, USA) or Gibco-BRL (Grand Island, NY, USA) unless otherwise indicated. HaCaT, CCD-986SK, and Raw264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified incubator under an atmosphere containing 5% CO_2.

Stimulation of Raw264.7 cells

To stimulate Raw264.7 cells, the cells (1×10^6/well) were plated to 96-well plates (Falcon, BD Biosciences, San Jose, CA, USA) in DMEM containing 10% FBS. The cells were incubated overnight, and then the medium was replaced with DMEM containing ramalin or 4 μg/ml dexamethasone in the presence or absence of 1 μg/ml *E. coli* lipopolyssacharide (LPS, serotype O111:B4) followed by further 24 h incubation. The culture medium was used for NO assay and the remaining cells were subjected to intracellular ROS (hydrogen peroxide) assay.

NO assay

Nitrite production was measured in culture medium. The medium (100 μl) was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphylenediamine dihydrochloride in 2.5% phosphoric acid) and incubated for 10 min at room temperature. The nitrite concentration was measured using the

Table 1
In vitro antioxidant capacities of ramalin and selected commercial standards.

Test samples	50% inhibition concentration (IC_{50})*				
	DPPH (μg/ml)	$ABTS^{\bullet+}$ (μg/ml)	Superoxide (μg/ml)	Tyrosinase (μg/ml)	Reducing power (1 μg of BHT equivalent)
Ramalin	0.99 ± 0.08	1.7 ± 0.2	10.2 ± 1.2	4 ± 0.4	0.4 ± 0.03
BHA	4.98 ± 0.9	–	–	–	–
Trolox	–	46.4 ± 5.1	–	–	–
Ascorbic acid	–	–	12.7 ± 1.2	–	–
Kojic acid	–	–	–	5 ± 0.5	–

*All the data were statistically significant with $p < 0.05$.

optical density at 540 nm. All the samples were analyzed in triplicate.

Measurement of intracellular ROS (hydrogen peroxide) production by flow cytometry

ROS generation in macrophage cells was detected by flow cytometric analysis, as described previously (Sinha et al. 2006). Briefly, 5×10^5 cells were plated to a culture dish and treated with LPS (with or without ramalin) for 24 h. Cells treated with LPS and ascorbic acid were used as a positive control. After the treatment, cells were collected and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA, Sigma) was added to the cell suspension at a final concentration of 10 μM. After incubating for 15 min in the dark at 37 °C, the cells were centrifuged and the pellet was washed twice with ice-cold PBS. The pellet was resuspended in PBS and the fluorescence intensity was analyzed by flow cytometry. DCF-DA fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. Intracellular ROS generation was analyzed on FACSCalibur flow cytometer (BD biosciences) using CellQuest software.

Cytotoxicity assay

The effect of ramalin on cell viability was determined by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay [Cell Counting Kit-8 (CCK-8), Dojindo laboratories, Japan]. First, 100 μl of cell suspension (1×10^4 cells) was dispensed in a 96-well plate and pre-incubated for 24 h in a humidified incubator (37 °C, 5% CO_2). Ten microliters of various concentrations of ramalin was then added to the plate and the mixture was incubated for 24 h (37 °C, 5% CO_2). Following incubation, 10 μl of CCK-8 solution was added to each well, and the plate was again incubated for 1 h. Absorbance was then measured at 420 nm using a microplate reader.

Statistical analysis

All data were expressed as mean ± SD from a minimum of three replicates. 50% inhibition concentration (IC_{50}) was calculated by linear regression analysis of the obtained data using Microsoft Excel. Analysis of variance was performed by ANOVA procedures and $p < 0.05$ was considered to be statistically significant.

Results

Antioxidant capacity (in vitro)

Various antiradical capacities of ramalin as well as commercial standard compounds are presented here (Table 1) in term of 50% inhibition concentration (IC_{50}). The experimental data showed that ramalin contained a 5-fold greater capacity to scavenge DPPH free radical than that of BHA and 27 times stronger activity to scavenge $ABTS^{\bullet+}$ radical than trolox. Similarly, ramalin was found 2.5 folds more potent activity than BHT and 1.2 times stronger capacity than ascorbic acid in ferric ions reducing and superoxide anion scavenging assays, respectively. In addition, ramalin was found to be 1.25 times more powerful than kojic acid to inhibit tyrosinase activity.

Antioxidant capacity (in vivo)

NO and intracellular ROS (hydrogen peroxide) assays

Murine macrophage Raw264.7 cells were stimulated by LPS, and >1 μg/ml ramalin reduced the released NO significantly (Fig. 2). The cells were stimulated by LPS and the produced intracellular ROS (hydrogen peroxide) was dramatically diminished by treating

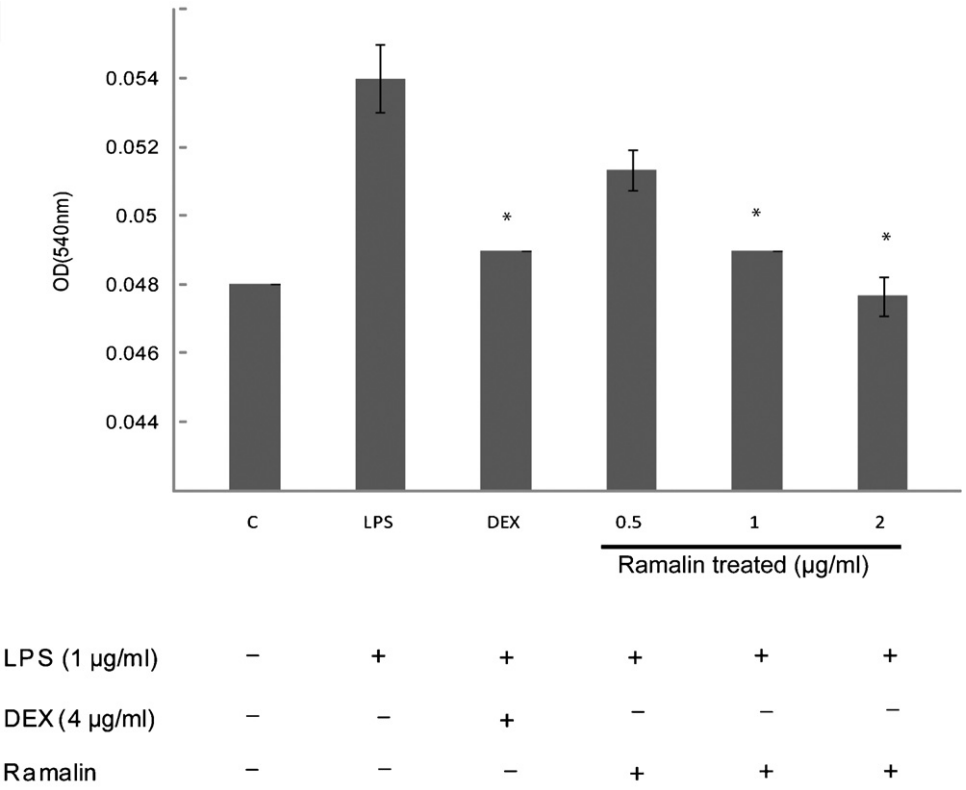

Fig. 2. The activity of ramalin to reduce NO generated by murine macrophage Raw264.7 cells when activated with LPS. Dexamethasone (Dex.) was used as a positive control. Asterisks (*) denote $p < 0.05$. These data represent the mean ± 1 S.E. of three replicates. + indicated treated and − indicated untreated.

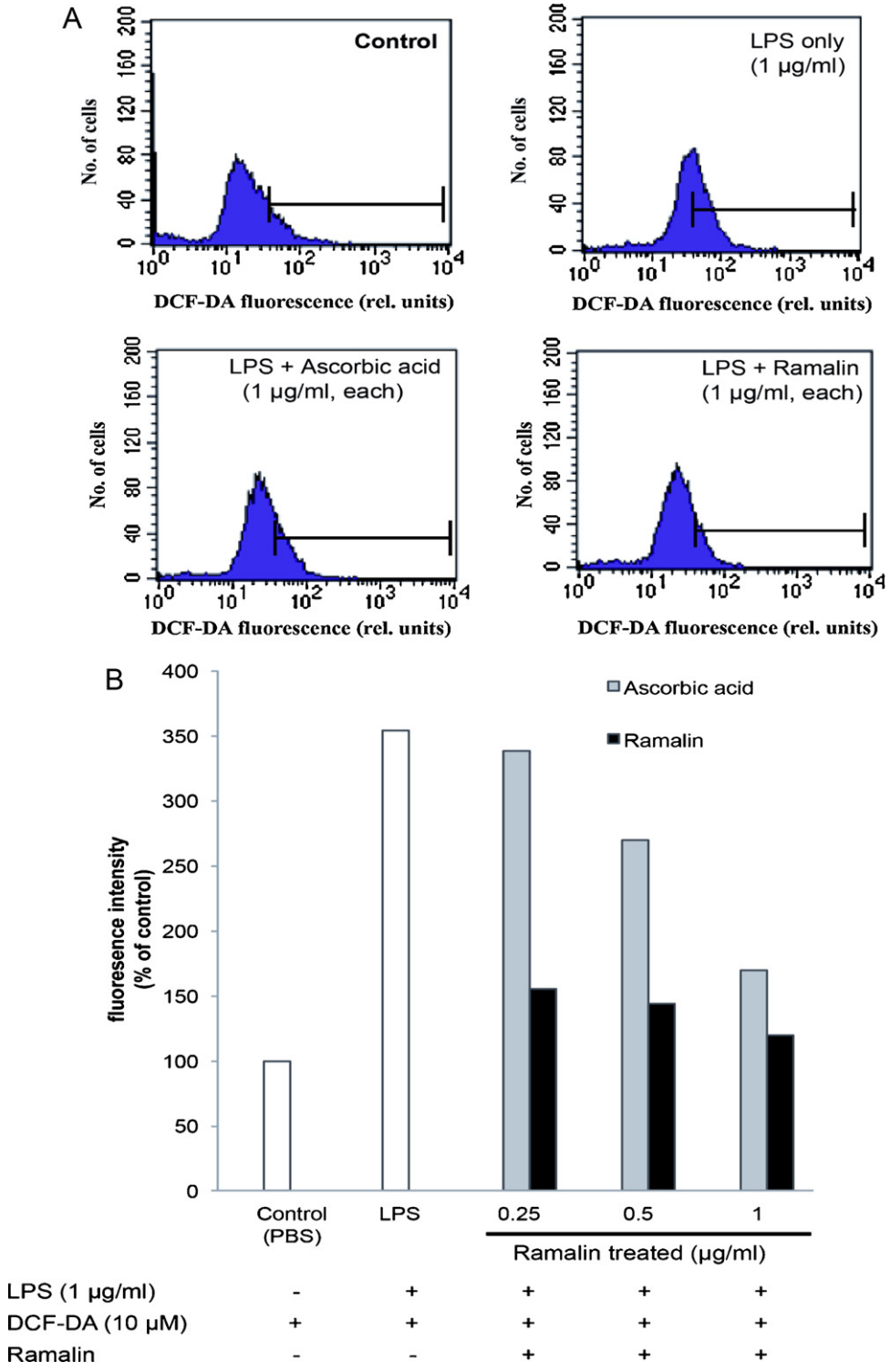

Fig. 3. The effect of ramalin on reactive oxygen species (ROS) production in Raw264.7 cells. (A) Representative FACS histograms depicting ROS-dependent DCF-DA fluorescence in Raw264.7 cells. Cells with no treatment were used as a control. Cells stimulated with LPS (1 μg/ml) for 24 h in the absence and presence of ascorbic acid or ramalin were shown. Fluorescence intensities were measured using CellQuest software. (B) Bar graphs showing the quantitative FACS data.

>0.25 μg/ml ramalin in a concentration-dependent manner, which was more effective than 1 μg/ml ascorbic acid treatment (Fig. 3).

Cytotoxicity of ramalin

The toxic effect of the ramalin was analyzed in human fibroblast (CCD-986SK) cells and keratinocyte (HaCaT) cells using the MTT reduction assay. The treatment with increasing doses of ramalin during 24 h showed dose-dependent cytotoxicity. The IC_{50} for the cell viability of CCD-986SK cells and HaCaT cell were 28 μg/ml and 25.9 μg/ml, respectively.

Discussion

To identify natural antioxidants from lichen species from Antarctica, extracts from 11 lichen species were collected for the present investigation. Three antioxidant assays based on the elec-

tron transfer (ET) system (DPPH free radical, $ABTS^{\bullet+}$ and Fe^{+3} reducing power) and one antioxidant assay system against biologically relevant oxidant [superoxide (O_2^-) anion] were used to evaluate the antioxidant capacities of the extracts from the Antarctic lichens. In addition, mushroom tyrosinase-inhibition capacity of the lichens extracts was also investigated. Preliminary screening result showed that extract from *Ramalina terebrata* exhibited comparable antioxidant activity against various commercial standard compounds (data not shown). Therefore, mass extraction of *R. terebrata* was performed to identify the active antioxidant principles in the extract, followed by the application of various chromatographic techniques. As a result, the principal active ramalin (Fig. 1) was isolated from the extract from *R. terebrata* extract, and the structure was identified by analysis of spectroscopic data (HRESIMS, ^{1}H NMR, ^{13}C NMR, HMBC, and HMQC).

DPPH is a stable free radical in aqueous or methanol solution and becomes a stable molecule by accepting an electron or hydrogen radical. DPPH is commonly used to measure the antioxidant activity of extracts or pure compounds (Duh et al. 1999). *In situ*, there are various free radicals, such as polyaromatic hydrocarbon cations, which are associated with carcinogenesis. Thus, compounds that will scavenge DPPH *in vitro* may also scavenge radicals like polyaromatic hydrocarbon cations *in vivo*. In this study, ramalin scavenged DPPH free radicals, showing potency five times higher than that of commercial BHA. Thus, from this data, it could be concluded that ramalin had strong hydrogen-donating capacity.

Similarly, the $ABTS^{\bullet+}$ scavenging capacity of ramalin was also tested. In this reaction system, chromogen cations of ABTS are produced by oxidizing ABTS with ferryl myoglobin radicals. Ferryl myoglobin radicals are produced by the reaction of metmyoglobin with hydrogen peroxide. The ABTS free radical produces a green color and can be read spectrophotometrically at 405 nm (Rice-Evans and Miller 1994). Like trolox, ramalin also reduced the production of $ABTS^{\bullet+}$ radicals in the reaction mixture in a concentration-dependent manner. Linear regression analysis of the data showed that the $ABTS^{\bullet+}$ scavenging capacity of ramalin was approximately 27 times greater than that of trolox.

It has been observed that the reducing power of a compound directly correlates with its potential antioxidant activity (Duh et al. 1999; Meir et al. 1995). The reducing activities of compounds are based on the presence of reductones, which exert antioxidant effects by donating a hydrogen atom to break the free radical chain (Gordon 1990). The ferric ion reducing antioxidant (or reducing power) assay measures the electron transferring capacity of the test sample to convert Fe^{3+} to Fe^{2+} inside a complex molecule. In the present experiments, the reducing power of ramalin was compared to that of the antioxidant, BHT. The results showed that ramalin contained high reducing capacity. With the available experimental data, it was calculated that ramalin *was* 2.5 times more potent in reducing capacity than BHT.

Superoxide radicals, a precursor of other reactive oxygen species, are very harmful to cellular components (Halliwell and Gutteridge 1990). Large amounts of superoxides can be produced inside the body during various metabolic and physiological processes (Gülçin et al. 2004; Blaszczky et al. 1994; Bedard et al. 2001). The superoxide (O_2^-) scavenging capacity of ramalin was measured by using a riboflavin NBT-light system *in vitro*. In the riboflavin-NBT light system, photochemical reduction of flavin generates O_2^- radicals, which induce NBT, causing the formation of blue formazan (Beauchamp and Fridovich 1971). It was found that the (O_2) scavenging capacity of ramalin increased in a dose-dependent manner and that ramalin inhibited the formation of the blue formazan more strongly than the antioxidant ascorbic acid.

Tyrosinase is a multifunctional copper-containing enzyme that catalyses the oxidation of monophenols, O-diphenols, and O-quinones (Azhar-ul-Haq et al. 2006). Tyrosinase is known to be a key enzyme for melanin biosynthesis (Baurin et al. 2002). Melanin synthesis in animals proceeds from L-tyrosine through a series of enzymatic and chemical steps initiated by tyrosine hydroxylation to yield L-DOPA, and subsequent L-DOPA oxidation to L-DOPA quinone. Tyrosinase is a single enzyme that catalyses both reactions (Okunji et al. 2007). Thus, tyrosinase inhibitory compounds are useful for the treatment of some dermatological disorders associated with melanin hyperpigmentaion. In addition, these compounds are also useful in the cosmetics industries for whitening and depigmentation after sunburn (Azhar-ul-Haq et al. 2006). Melanin is a heteropolymer of indole compound and produced inside melanocytes cells, which contain tyrosinase enzyme (Robb 1984). Other factors, as metal ions and the TRP-1 and TRP-2 enzymes also have some role for the production of melanin but, tyrosinase enzyme has a major regulatory role in melanin biosynthesis (Azhar-ul-Haq et al. 2006). Thus, many tyrosinase inhibitors compounds that suppress melanogenesis have been widely studied to develop the technique for the treatment of hyperpigmentation (Masamoto et al. 2003). In this study, tyrosinase inhibitory activity of ramalin was investigated and compared with the tyrosinase inhibitor, kojic acid. The experimental results showed that ramalin had more potent inhibition of tyrosinase than that of kojic acid.

The cytotoxic effect of ramalin on human fibroblast cell and keratinocyte cells was investigated by the MTT assay. MTT is a yellow water-soluble tetrazolium salt and viable cells are only able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium rings (Kilani et al. 2008). Because this occurs mostly in the mitochondria, the assays largely measure mitochondrial activity. Thus, this assay is used to determine cytotoxicity of potential medicinal agents and other toxic materials. Those agents which cause cell toxicity lead to mitochondrial dysfunction and therefore decreased performance in the assay.

The IC_{50} concentration of ramalin in various *in vitro* and *in vivo* antioxidant assays discussed here was found to be below the toxicity level of the compound to human cell lines. Such results clearly indicated that ramalin is very strong non-toxic antioxidant compound with enough potential to be used in future therapeutic applications.

Excessive ROS are associated with cell damage and the body has developed defense mechanisms to minimize free radical-induced cell damage. Antioxidants play a key role in these mechanisms. One microgram per milliliter ramalin significantly reduced the released NO and 0.25 μg/ml ramalin diminished the generated intracellular ROS in LPS-stimulated murine macrophage Raw264.7 cells (Fig. 3). Macrophage cells are a part of critical defense mechanisms of immune system, but cause a number of problems when the cells are abnormally stimulated to induce excessive oxidative stress. Therefore, ramalin may serve as ROS regulator.

The antioxidant properties of polyphenols are due to the presence of their many phenolic hydroxyl groups, which have high potential for scavenging free radicals (Hatano et al. 1989; Sawa et al. 1999). Phenolic compounds donate hydrogen to reactive radicals and break the chain reaction of lipid oxidation at the initiation step (Gülçin et al. 2004). For the last decade, natural antioxidants, especially phenolic compounds, have been used for the treatment of various diseases such as neurodegenerative diseases, cancer, diabetes, cardiovascular dysfunctions, inflammatory diseases, and aging. The use of phenolic compounds in the field of medicine is due to their antioxidant capacity, free radical scavenging capacity, chelation of redox active metal ions, modulation of gene expression, and interaction with the cell signaling pathway (Soobrattee et al. 2005). It was also suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans (Tanaka et al. 1998). The antioxidant and free radical scavenging activities of phenolic compounds are dependent upon the arrangement of functional groups around the nuclear structure. Thus, both

the number and configuration of H-donating hydroxyl groups are the main structural features that influence the antioxidant capacity of phenolics (Cao et al. 1997; Pannala et al. 2001).

Considering the molecular structure of ramalin (Fig. 1), the two hydroxyl groups from the phenolic and carboxylic acid functionalities in the molecule could donate hydrogen to scavenge free radicals. Similarly, three amino groups can donate their free electrons to the reactive ions, making them stable. Thus, the more potent antioxidant activity of ramalin compared to the other antioxidants tested could be attributed to the presence of several hydrogen and electron donating groups within the single molecule. In addition, the lack of toxicity of ramalin against two human cell lines, within the range of working concentrations, suggests that ramalin could be viewed as a potential product for future cosmetic and therapeutic applications. Furthermore, the present study shows that ramalin has significant antioxidant activity indicating its high scavenging activity against nitric oxide and hydrogen peroxide free radicals in murine macrophage cells. Consequently, we suggest that ramalin can be a strong therapeutic candidate for controlling oxidative stress in cells. Further research on the mass production of ramalin and toxicological evaluation against other human cell lines are strongly warranted.

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