Effects of Pro-Arg, a Novel Dipeptide Derived from Protamine Hydrolysate on H₂O₂-Induced Oxidative Stress in Human Diploid Fibroblasts

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Oxidative stress plays an important role in tissue damage associated with many different chronic degenerative diseases, such as cancer and cardiovascular disorders. In our previous study, a novel dipeptide Pro-Arg derived from protamine hydrolysates was found to possess significant antioxidative activity *in vitro*. In this study, we compared the antioxidant capabilities of purified and synthesized dipeptide (Pro-Arg) and found both of them had very strong hydroxyl-radical scavenging activity *in vitro*, even at a very low concentration. The protective effect of the dipeptide against oxidative stress was evaluated using H₂O₂-induced oxidative stress of human diploid fibroblasts MRC-5 cell model. Our results showed the dipeptide attenuated H₂O₂-induced cellular oxidative damage, and normalized S phase arrest of MRC-5 cells exposed to H₂O₂. Our findings demonstrate that Pro-Arg can protect against oxidative stress/damage and H₂O₂-induced human diploid fibroblasts cell death.

Key words antioxidant dipeptide; human diploid fibroblast; reactive oxygen species

Oxidation of biomolecules has been identified as a free radical-mediated process, which causes many unfavorable impacts on food and biological systems. The use of antioxidants is one of the effective control methods against oxidation.^{1–3)} It has been reported that dietary antioxidants can scavenge reactive oxygen species (ROS) in the body, lowering oxidative stress involved in tissue damage, and decelerating aging and chronic degenerative diseases, such as cancer and cardiovascular disorders.^{4–6)}

For more than a century, natural oligopeptides have attracted scientific attention.^{7–8)} Oligopeptides differ substantially from larger polypeptides (proteins) in their physicochemical and biological properties. The smaller peptides rarely possess strong enough intramolecular attractions to form stable globules.⁹⁾ Therefore, they are able to shift configurations and to fit themselves into specific receptor molecules, a process which is further aided by high diffusional mobility.^{10,11)} Recently, more reports have focused on dipeptide because of these advantages. Firstly, many of dipeptides can be administered in a smaller volume because of their better solubility compared with the individual amino acids. Secondly, dipeptides are better absorbed than individual amino acids.^{12,13)} Finally, the osmolality of the dipeptides is lower than the amino acids administered individually.¹⁴⁾

The first reported oligopeptide, carnosine, which is an endogenous dipeptide in animal tissues such as skeletal muscle, has been shown to be an effective antioxidant.^{15–18)} Since that time, thousands of oligopeptide regulators have been described.⁸⁾ In our previous studies, protamine hydrolysate was found to possess significant antioxidative activity both *in vitro* and *in vivo*.¹⁹⁾ Peptide fractions obtained from protamine hydrolysate exhibited higher antioxidant activity against free radicals in oxidation system at low concentrations.²⁰⁾ In this study, we investigated the scavenging effect of dipeptide Pro-Arg on hydroxyl radical and its cytoprotective effect against hydrogen peroxide (H₂O₂)-induced oxidative stress in human diploid fibroblast, MRC-5 cell line.^{21,22)}

MATERIALS AND METHODS

Materials Salmon protamine hydrolysate was produced by Wuhan Tallyho Peptide Institute (Wuhan, China). Human fetal lung diploid fibroblast (MRC-5) was obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences (Beijing, China). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and propidium iodide (PI) were purchased from Sigma (St. Louis, U.S.A.). Deoxyribose was purchased from Fluka (St. Louis, U.S.A.). Symmetry Shield™ RP18 (3.9×150 mm) column was from Waters (Milford, U.S.A.).

Preparation of Antioxidative Peptide The concentration of protamine hydrolysate was determined according to the method of Lowry *et al.*²³⁾ The dipeptide responsible for strongest antioxidant activities was obtained from protamine hydrolysate, and then identified by LC/MS and the database search in NCBI.²⁰⁾

The antioxidant dipeptide was synthesized by Shanghai ShineGene Company using solid phase peptide synthesis methods. Purity was determined using an symmetry Shield™ RP18 (3.9×150 mm) column with a liner gradient of acetonitrile (5–15% in 40 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min on Waters 2695 Separation Module system with a PDA (photo diode array) detector. The PDA detector was monitored at 254 nm. The molecular mass of the isolated peptide was determined by mass spectrometry on PE Biosystems Voyager DE MALDI-TOF (ABI, Austin, U.S.A.).

Scavenging Activity on Hydroxyl Radical According to the method of Halliwell *et al.*²⁴⁾ with a slight modification, a total volume of 1 ml reaction mixture containing samples, pH 7.4 KH₂PO₄–KOH buffer, 1.04 mM ethylenediaminetetraacetic acid (EDTA), 1 mM FeCl₃, 12 mM H₂O₂, 60 mM deoxyribose and 1 mM vitamin C was added into a test-tube with a glass stopper and then incubated at 37°C for 1 h. After the completion of incubation, 1 ml of 25% HCl and 1 ml of 0.5% thiobarbituric acid solution were in turn added quickly. The final mixture was boiled for 15 min and then

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cooled with tap water. The absorbance of the mixture was determined at 532 nm on a Thermo Multiskan MK3 microplate reader. The hydroxyl radical-scavenging activity was calculated as follows:

$$\text{scavenging activity (\%)} = \frac{A_0 - (A_1 - A_2)}{A_0} \times 100\%$$

Where A_0 indicates the absorbance of the blank (distilled water instead of samples); A_1 , the absorbance of the mixture containing samples; A_2 , the absorbance of the mixture without deoxyribose.

Cell Culture Human fetal lung diploid fibroblasts (MRC-5) were cultured in Dulbecco's modified eagle's medium (DMEM) (Invitrogen, Carlsbad, U.S.A.) supplemented with 10% fetal calf serum, 100 units/ml of penicillin and 100 units/ml of streptomycin at 37°C in a humidified atmosphere of 95% O₂ and 5% CO₂. The cells were fed every other day and passaged at a ratio of 1:3 before the cultures were confluent. The trypan blue dye exclusion method was used to ensure that an equal number of viable cells were seeded during subculture.

Detection of ROS by Dichlorofluorescein (DCF) Staining Formation of reactive oxygen species (ROS) in cells was detected by monitoring the fluorescence of DCF, the oxidation product of DCFH-DA.²⁵ MRC-5 cells were seeded into 96-well plates at density of approximately 2×10^5 cells/well for 2 h pre-culture, and then treated with the dipeptide in distilled water for 24 h (final concentrations of dipeptide were 0, 25, 50, 100, 250 µg/ml, respectively). Afterwards the cells were exposed to 100 or 300 µM H₂O₂ solutions in DMEM for 1 h, and then washed twice with phosphate buffered solution (PBS). Then, the cells were loaded with 100 µM DCFH-DA for 30 min at 37°C. After the treatment, they were immediately re-fed with fresh serum-free medium, and measured by fluorescent plate reader (BMG Labtech NOVOstar, Offenburg, Germany).

Cell Cycle Analysis by Flow Cytometry Cell cycle analysis was performed by flow cytometry based on DNA content. MRC-5 cells (1×10^6 cells per well) were seeded on a 6-well plate and cultured for 2 h in DMEM. After exposure

to 300 µM H₂O₂ for 3 h and 6 h, the cells were collected, rinsed with ice-cold PBS, trypsinized, and resuspended in 0.3 ml of PBS. They were fixed and permeabilized by addition of 0.7 ml of ethanol. After a brief wash, the cells were gently resuspended in 1 ml of propidium iodide (PI) stain solution (500 µg/ml propidium iodide, 500 µg/ml RNase A in PBS), and incubated at room temperature for 30 min before analysis on a flow cytometer using Cell Quest software (BD FAC-SCalibur, San Jose, U.S.A.).

For analysis of the effects of dipeptide on cell cycle, exponentially growing cells were seeded on a 6-well plate and incubated for 2 h followed by addition of dipeptides to the final concentrations of 0–125 µg/ml. After 24 h incubation, the cells were treated with 300 µM H₂O₂. After 6 h treatment, cell cycle distribution was analyzed as described above.

Statistical Analysis Data are presented as the mean ± S.D. from at least three independent experiments and analyzed using Student's *t*-test. Differences with a *p*-value < 0.05 were considered statistically significant.

RESULTS

Preparation of the dipeptide Pro-Arg Through consecutive chromatographic methods, the peptide obtained with the highest antioxidative activity was identified to be Pro-Arg matching 1–2 and 16–17 residues of the salmon protamine.²⁰ To further investigate the antioxidative activities of the dipeptide Pro-Arg, the dipeptide was synthesized. HPLC profile of the synthesized dipeptide was shown in Fig. 1, and the purity of the dipeptide is higher than 98.74%.

Hydroxyl Radical Scavenging Activity of the Dipeptide Pro-Arg As shown in Table 1, the activities of the dipeptide were higher than those of mannitol, and the activities of the synthesized dipeptide were higher than those of the purified dipeptide. Meanwhile, the dipeptide displayed dose-dependent scavenging activity on the hydroxyl radical. These results indicated that the dipeptide had very strong hydroxyl-radical scavenging activity *in vitro*, even at a very low concentration.

Effect of the Dipeptide on ROS As shown in Fig. 2, in-

Fig. 1. Purity Analysis of the Synthesized Dipeptide by HPLC

Table 1. The Scavenging Activity of the Dipeptide against Hydroxyl Radical

Samples	Concentrations ($\mu\text{g/ml}$)	Scavenging activity (%)
Synthesized dipeptide	5	19.88 $\pm$ 2.45
	10	31.86 $\pm$ 0.51
	20	37.29 $\pm$ 5.03
	40	48.89 $\pm$ 2.51
	100	64.10 $\pm$ 3.02
Purified dipeptide	10	3.51 $\pm$ 0.57
	20	12.89 $\pm$ 1.21
	40	21.04 $\pm$ 0.77
	100	53.23 $\pm$ 1.71
	5000	20.17 $\pm$ 1.51
Mannitol	10000	39.84 $\pm$ 2.11
	20000	71.87 $\pm$ 1.09
	40000	88.42 $\pm$ 3.29

IC₅₀ values of mannitol, purified dipeptide and synthesized dipeptide against hydroxyl radical were 11.85 mg/ml, 91.38 $\mu\text{g/ml}$ and 41.11 $\mu\text{g/ml}$ respectively.

Fig. 2. Dose-Dependent Effects of the Dipeptide on Intracellular ROS

Intracellular ROS production in MRC-5 cell cultures preincubated with the synthesized dipeptide for 24 h and then exposed to H₂O₂ for 1 h. Black (in 100 μM H₂O₂ experiment group) and white columns (in 300 μM H₂O₂ experiment group) indicated intracellular basic ROS concentration of MRC-5 cells without H₂O₂ treatment; various concentrations (250, 100, 50, 25, 0 $\mu\text{g/ml}$ in X-axis) of the dipeptide were used to pre-treat MRC-5 cells. (* p <0.05 compared with the 0 $\mu\text{g/ml}$ dipeptide). Control, cells were not challenged with H₂O₂.

tracellular ROS concentrations in MRC-5 cells treated with 300 μM H₂O₂ were normally higher than those treated with 100 μM H₂O₂, regardless of the addition of dipeptide or not. Exposure of MRC-5 cells to 300 μM H₂O₂ initiated a significant increase in ROS level, which was 1.43-fold of that of control cells. These results demonstrated that the H₂O₂-induced intracellular ROS model was successful.

Dipeptide pre-incubation caused a dose-dependent decrease of intracellular ROS concentration. ROS level in cultures pre-incubated with 250 $\mu\text{g/ml}$ dipeptide was significantly decreased by 30% (p <0.05), compared with that of 0 $\mu\text{g/ml}$ dipeptide pre-incubation. These results showed that dipeptide pretreatment could eliminate about 90% intracellular ROS induced by H₂O₂ when the concentration of synthesized dipeptide reached 250 $\mu\text{g/ml}$.

Effect of H₂O₂ and the Dipeptide on Cell Cycle MRC cells treated with H₂O₂ for 3 h and 6 h were characterized by a significant decrease in the fraction of G₂/M phase (Fig. 3). Concomitant with such change there was an over 1.15-fold

Table 2. Effects of Synthesized Dipeptide on Cell Cycle

	Percentage of cells in each phase of cell cycle (%)		
	G ₀ /G ₁	S	G ₂ /M
None	45.79 $\pm$ 1.06	28.07 $\pm$ 1.07	26.14 $\pm$ 2.14
H ₂ O ₂ (300 μM)	43.23 $\pm$ 1.25	41.17 $\pm$ 0.81*	15.60 $\pm$ 0.44*
H ₂ O ₂ (300 μM) + dipeptide (25 $\mu\text{g/ml}$)	44.49 $\pm$ 1.41	37.72 $\pm$ 0.77 [#]	17.79 $\pm$ 1.04
H ₂ O ₂ (300 μM) + dipeptide (125 $\mu\text{g/ml}$)	46.18 $\pm$ 0.92	30.57 $\pm$ 0.38 [#]	23.25 $\pm$ 0.57 [#]

MRC-5 cells were preincubated with synthesized dipeptide for 24 h and then exposed to 300 μM H₂O₂ for 6 h. Compared with Control group (None), * p <0.05; Compared with mode group (300 μM H₂O₂), [#] p <0.05.

and 1.47-fold increase in the portion of S cells. In cells treated with H₂O₂ for 6 h, the S-phase population reached 41%, increasing by 13% compared with untreated MRC-5 cells. Simultaneously, the G₂/M-phase population was decreased by 11%. The results showed that MRC-5 cells accumulated in S phase were in response to induction by H₂O₂ in a time-dependent manner.

As shown in Table 2 and Fig. 3, dipeptide pretreatment had apparent dose-dependent effects on abnormal cell cycle distribution induced by 300 μM H₂O₂ treatment for 6 h. It reduced S-phase population from 41 to 37%, and increased G₂/M-phase population from 15 to 17% when the concentration reached 25 $\mu\text{g/ml}$. The negative effects induced by H₂O₂ were almost eliminated by the dipeptide at 125 $\mu\text{g/ml}$.

DISCUSSION

Recently, many reports have shown that hydrolyzed peptides could possess high antioxidant activities.^{26,27)} Peña-Ramos *et al.* reported that the antioxidant ability of various peptide fractions was based on peptide size and amino acid composition.²⁸⁾ Some amino acids with antioxidant activity, such as histidine, proline, tyrosine and lysine, were abundant in most of the peptide fractions that exhibited good antioxidant activity.^{29,30)} Arginine, a diamine monocarboxylic amino acid with a guanidinium group, is a precursor in the formation of nitric oxide. Supplemental L-arginine has been proved to have the antioxidative effect.^{31,32)} Chen *et al.* reported that the addition of Pro to the N-terminus of His-His dramatically increased its activity and Pro-His-His with the N-terminus of Pro was the strongest antioxidant among all 28 synthetic peptides.³³⁾ In addition, L-Arg has some protective functions against ROS attack possibly because of its direct chemical property interaction with superoxide anion radical.³⁴⁾ It suggests the antioxidant mechanism of L-Arg is involved in that of the dipeptide Pro-Arg. Thus, the antioxidant activity of the dipeptide may attribute to specific peptide size, specific peptide bond and the presence of Pro and Arg. It was reported that high hydroxyl radical scavenging activity of peptide-containing organic molecules has been explained on the basis of their susceptibility to oxidation by extremely reactive hydroxyl radicals and on the chelation of Fe²⁺, which retards the generation of hydroxyl radicals by the Fenton reaction.³⁵⁾ In the method we used to test scavenging activity of dipeptide on hydroxyl radical, the initial products of OH $\cdot$ attack on deoxyribose is very complex and iron-EDTA

Fig. 3. Time-Dependent Effects of H_2O_2 and Protective Effect of the Dipeptide on Cell Cycle Analyzed by Flow Cytometry

(A) Cell cycle distribution of MRC-5 cells untreated with H_2O_2 ; (B) Cell cycle distribution of MRC-5 cells treated with $300 \mu\text{M}$ H_2O_2 for 3 h; (C) Cell cycle distribution of MRC-5 cells treated with $300 \mu\text{M}$ H_2O_2 for 6 h. (D) Cell cycle distribution of MRC-5 cells pre-treated with $125 \mu\text{g/ml}$ dipeptide for 24 h, followed by $300 \mu\text{M}$ H_2O_2 treatment for 6 h.

complex may be involved not only in the generation of $\text{OH}^\cdot$ but also in later stage of the reaction. This assay is inapplicable to strong metal-chelating compounds that can withdraw iron from EDTA.²⁴⁾ Carnosine is a typical antioxidative dipeptide, and its IC_{50} value on hydroxyl radical was about 8.9 mM as previously determined.³⁶⁾ In this study, IC_{50} value of the synthesized dipeptide against hydroxyl radical was 0.15 mM . Our data demonstrated that the dipeptide had better antioxidative activity *in vitro* compared with carnosine.

Reactive oxygen species (ROS) includes both the radical and nonradical species that participate in the initiation and propagation of radical chain reactions. Accumulation of ROS beyond the immediate needs of the cell may affect cellular structure and functional integrity, by bringing about oxidative degradation of critical molecules, such as the DNA, proteins, and lipids. Antioxidants can exert positive pharmacological effects on specific human diseases by ameliorating the harmful effects of ROS. Our study suggests that the dipeptide is a primary antioxidant responsible for neutralizing ROS that induces cellular damage and exhibit a cytoprotective effect.

Hydrogen peroxide was proven to cause oxidative stress characterized by cell cycle arrest. Munoz *et al.* reported that H_2O_2 , which inhibits bromodeoxyuridine (BrdU) incorporation during DNA synthesis in a dose-dependent manner, has an inhibitory effect on cell cycle progression in the G_1 phase.³⁷⁾ Xu *et al.* investigated the novel mechanism of G-rich oligonucleotides (GRO) activity on nucleic acid and protein biosynthesis. GRO showed an arrest effect of the cell cycle predominantly in S phase.³⁸⁾ Joe *et al.* found resveratrol

caused an increase of cells in the S phase and a corresponding decrease of cells in the G_1 and $\text{G}_2\text{-M}$ phases in HCE7, MCF7, SW480, and HL60 cells.

But in Bic-1 cell, resveratrol treatment led to G_1 -phase arrest, and resveratrol did not appear to alter the cell cycle distribution in Seg-1 cell.³⁹⁾ Some reports suggested that in their *in vivo* model, lower cellular proliferation rates and host factors, including immune system-mediated events, might explain this difference.⁴⁰⁾ In our experiments, H_2O_2 treatment caused significant cell cycle arrest in S phase on MRC-5 cell. Host factor and cellular status may play important roles. Although, we can not differentiate among these different possibilities, since H_2O_2 presents a broad oxidative activity, it is possible that H_2O_2 is responsible for different effects at different locations in the cell. Moreover, we can not differentiate whether the effects are direct or indirect (*e.g.*, *via* the production of other ROS such as hydroxyl radical or lipid peroxides, or *via* secondary processes, such as the stimulation of intracellular calcium release). Previously reported indicated dipeptides can enter cells both *in vitro* and *in vivo*.^{41,42)} Therefore, this dipeptide, Pro-Arg, may enter the cultured cells. However, whether it can enter cells fully or partially needs further investigation.

Aging is an inevitable physiological phenomenon caused by several factors. Proliferative cells such as human diploid fibroblasts, displayed typical replicative senescence. In this study, we used MRC-5 human diploid fibroblasts (HDFs) with H_2O_2 -induced oxidative damage as cellular aging model, which was first described by Hayflick and Moorhead

to study aging-associated molecular changes in human cells.²¹⁾ Okada *et al.* reported that antioxidants with free radical-scavenging activity delayed cellular aging-dependent degeneration by increasing glutathione and catalase activities in human fibroblasts.⁴³⁾ It was reported that Kangen-karyu extract showed strong anti-aging effects on H₂O₂-treated HDFs due to its antioxidative activity and carnosine can delay aging and rejuvenate senescent cultured human fibroblasts.^{22,44)} Our present data showed that S phase arrest of MRC-5 cells was caused by H₂O₂-induced oxidative stress, whereas treatment with the dipeptide attenuated the oxidative status and normalized the cell cycle by decreasing the proportion of cells in the S phase, implying a correlation between a quiescent state of cells, such as growth arrest, and the antioxidant defense system. In a sense, our results suggested that the dipeptide may delay the aging process by virtue of its antioxidative effects.

In summary, the new dipeptide is a strong antioxidant against hydroxyl radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion radical, and it protects the cells from oxidative challenge, both *in vivo* and *in vitro*.^{19,20)} These findings strongly indicate that the new dipeptide is a good health care or drug candidate, and it is worthwhile to further characterize their pharmacological profiles in more complex cellular and animal models. It is anticipated that in the near future such targets will be related to various lifestyle-related disease groups, such as cardiovascular diseases, cancers, osteoporosis and obesity. Physiologically active peptides derived from protein hydrolysate offer a promising approach to prevent, control and even treat such diseases.

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REFERENCES

- Frankel E. N., *Food Chem.*, **57**, 51—55 (1996).
- Nam K. C., Ahn D. U., *Meat Sci.*, **63**, 1—8 (2003).
- Bekhit A. E. D., Geesink G. H., Ilian M. A., Morton J. D., Bickerstaffe R. C., *Food Chem.*, **81**, 175—187 (2003).
- Ferro-Luzzi A., Branca F., *Am. J. Clin. Nutr.*, **61**, 1338—1345 (1995).
- Serafini M., Bellocco R., Wolk A., Ekstrom A. M., *Gastroenterology*, **123**, 985—991 (2002).
- Vinson J. A., Liang X., Proch J., Hontz B. A., Dancel J., Sandone N., *Adv. Exp. Med. Biol.*, **505**, 113—122 (2002).
- Sewald N., Jakubke H. D., "Peptides: Chemistry and Biology," Wiley-VCH Verlag, GmbH, Weinheim, 2002.
- Zamyatnin A. A., Borchikov A. S., Vladimirov M. G., Voronina O. L., *Nucleic Acids Res.*, **34**, D261—D266 (2006).
- Privalov P. L., *Biophysics (Moscow)*, **30**, 722—733 (1985).
- Karle I. L., "The Peptides: Analysis, Synthesis," ed. by Gross E., Meienhofer J., Academic Press, NY, 1981, pp. 1—54.
- Zamyatnin A. A., *Biophysics (Moscow)*, **48**, 950—958 (2003).
- Silk D. B., Fairclough P. D., Clark M. L., Hegarty J. E., Marrs T. C., Addison J. M., Burston D., Clegg K. M., Matthews D. M., *J. Parenter. Enteral. Nutr.*, **4**, 548—553 (1980).
- Matthews D. M., *Physiol. Rev.*, **55**, 537—608 (1975).
- Neu J., Afzal A., Pan H., Gallego E., Li N., Calzi S. L., Caballero S., Spoerri P. E., Shaw L. C., Grant M. B., *Invest. Ophthalmol. Vis. Sci.*, **47**, 3151—3155 (2006).
- Lee B. J., Hendricks D. G., *J. Food Sci.*, **62**, 931—934 (1997).
- Sanchez-Escalante A., Djenane D., Torrecano G., Gimenez B., Beltran J. A., Roncales P., *Meat Sci.*, **64**, 59—67 (2003).
- Boldyrev A. A., Dupin A. M., Batrukova N. I., Bavykina N. I., Korshunova G. A., Shvachkin Y. P., *Comp. Biochem. Physiol. Part B*, **94**, 237—240 (1989).
- Decker A. E., Crum A. D., Calvert J. T., *J. Agric. Food Chem.*, **40**, 756—759 (1992).
- Wang Y. G., Zhu F. R., Han F. S., Wang H. Y., *Food Science and Technology (Chinese)*, **32**, 245—248 (2007).
- Wang Y. G., Zhu F. R., Han F. S., Wang H. Y., *J. Food Biochem.*, **32**, 654—671 (2008).
- Hayflick L., Moorhead P. S., *Exp. Cell Res.*, **25**, 585—621 (1961).
- Satoh A., Yokozawa T., Tanaka T., Okamoto T., Sei Y., *J. Trad. Med.*, **21**, 87—93 (2004).
- Lowry O. H., Rosebrough A. L., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- Halliwel B., Gutteridge J. M. C., Aruoma O. I., *Anal. Biochem.*, **165**, 215—219 (1987).
- Wang H., Joseph J. A., *Free Radic. Biol. Med.*, **27**, 612—616 (1999).
- Chen H. M., Muramoto K., Yamauchi F., Fujimoto K., Nokihara K., *J. Agric. Food Chem.*, **46**, 49—53 (1998).
- Kim S. K., Kim Y. T., Byun H. J., Nam K. S., Joo D. S., Shahidi F., *J. Agric. Food Chem.*, **49**, 1984—1989 (2001).
- Peña-Ramos E. A., Xiong Y. L., Arteaga G. E., *J. Sci. Food Agric.*, **84**, 1908—1918 (2004).
- Marcuse R., *Nature (London)*, **186**, 886—887 (1960).
- Kawashima K., Itoh H., Miyoshi M., Chibata I., *Chem. Pharm. Bull.*, **27**, 1912—1916 (1979).
- Jablecka A., Checinski P., Krauss H., Micker M., Ast J., *Med. Sci. Monit.*, **10**, CR29—CR32 (2004).
- Lin W. T., Yang S. C., Chen K. T., Huang C. C., Lee N. Y., *Acta Pharmacol. Sin.*, **26**, 992—999 (2005).
- Chen H. M., Muramoto K., Yamauchi F., Nokihara K., *J. Agric. Food Chem.*, **44**, 2619—2623 (1996).
- Lass A., Suessenbacher A., Wolkart G., Mayer B., Brunner F., *Mol. Pharmacol.*, **61**, 1081—1088 (2002).
- Rajapakse N., Mendis E., Jung W. K., Je Y. J., Kim S. K., *Food Res. Int.*, **38**, 175—182 (2005).
- Han L. Q., Yang G. Y., Wang Y. L., Wang Y. Y., *Journal of Henan University of Technology (Natural Science Edition) (Chinese)*, **27**, 43—46 (2006).
- Munoz C. M., van Meeteren L. A., Post J. A., Verkleu A. J., Verrips C. T., Boonstra J., *Free Radic. Biol. Med.*, **33**, 1061—1072 (2002).
- Xu X. H., Hamhouyia F., Thomas S. D., Burke T. J., Girvan A. C., McGregor W. G., Trent J. O., Miller D. M., Bates P. J., *J. Biol. Chem.*, **276**, 43221—43230 (2001).
- Joe A. K., Liu H., Suzui M., Vural M. E., Xiao D. H., Bernard Weinstein I., *Clin. Cancer Res.*, **8**, 893—903 (2002).
- Carbo N., Costelli P., Baccino F. M., Lopez-Soriano F. J., Argiles J. M., *Biochem. Biophys. Res. Commun.*, **254**, 739—743 (1999).
- Newey H., Smyth D. H., *J. Physiol.*, **164**, 527 (1962).
- Cheng B., Navab F., Lis M. T., Miller T. N., Matthews D. M., *Clin. Sci.*, **40**, 247—259 (1971).
- Okada Y., Okada M., *J. Nutr. Sci. Vitaminol.*, **46**, 1—6 (2000).
- Hipkiss A. R., Brownson C., Carrier M. J., *Mech. Ageing Dev.*, **122**, 1431—1445 (2001).