Scavenging of Reactive Oxygen Species by *Eriobotrya japonica* Seed Extract

Junko YOKOTA,*[,a] Daisuke TAKUMA,[a] Atsuhide HAMADA,[a] Masahide ONOGAWA,[b] Saburo YOSHIOKA,[a] Masahiko KUSUNOSE,[b] Mitsuhiko MIYAMURA,[b] Shojiro KYOTANI,[b] and Yutaka NISHIOKA[a,b]

[a] *Department of Biomedical Science, Kochi Medical Graduate School; and* [b] *Department of Pharmacy, Kochi Medical School Hospital; Kohasu, Oko-cho, Nankoku, Kochi 783–8505, Japan.*
Received September 29, 2005; accepted November 23, 2005; published online December 12, 2005

We have clarified that *Eriobotrya japonica* seed extract has strong antioxidative activity, and is effective for the prevention and treatment of various diseases, such as hepatopathy and nephropathy. In this study, to investigate the influences of components of *Eriobotrya japonica* seed extract on its antioxidative activity, extracts were prepared using various solvents (*n*-hexane (Hex), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH), methanol (MeOH) and H_2O) and the antioxidative activity of the solvent fractions and components was evaluated based on the scavenging of various radicals (DPPH and O_2^-) measured by the ESR method and the inhibition of Fe^{3+}-ADP induced NADPH dependent lipid peroxidation in rat liver microsomes. The radical scavenging activities and inhibitory activities on lipid peroxidation differed among the solvent fractions and components. In the *n*-BuOH, MeOH and H_2O fractions, radical scavenging activity and inhibitory activity on lipid peroxidation were high. In addition, these fractions contained abundant polyphenols, and the radical scavenging activity increased with the polyphenol content. In the low-polar Hex and EtOAc fractions, the radical scavenging activity was low, but the lipid peroxidation inhibition activity was high. These fractions contained β-sitosterol, and the inhibitory activity on lipid peroxidation was high. Based on these findings, the antioxidative activity of *Eriobotrya japonica* seed extract may be derived from many components involved in a complex mechanism, resulting in high activity.

Key words *Eriobotrya japonica*; ESR; natural antioxidant; LPO

Elucidation of the causal relationship between diseases and oxidative stress has recently progressed, and the close association of oxidation with the development and progression of not only life-style disorders, but also various intractable diseases and the aging process has been clarified.[1,2] The ingestion of antioxidative substances is useful for the prevention and treatment of these diseases, and various investigations have been performed.[3,4] *Eriobotrya japonica* has long been used as a pharmaceutical plant. Particularly, the leaves of *Eriobotrya japonica* LINDEL. are used in a traditional herbal remedy for skin diseases, inflammation, cough and expectoration. Leaves of *Eriobotrya japonica* LINDEL. have recently been shown to contain polyphenols, and a blood glucose-lowering action,[5—7] anti-inflammatory action[8,9] and anti-cancer action[10] have been reported. *Eriobotrya japonica* seeds have been reported to contain nitrile aromatic compounds, such as amygdalin,[11] and have been used as a substitute for An-nin in traditional herbal medicines. Nishioka *et al.* have recently reported that, in addition to aromatic compounds, *Eriobotrya japonica* seeds contain unsaturated fatty acids, such as linoleic acid and linolenic acid, and plant sterols, such as β-sitosterol,[12] and a 70% ethanol extract is effective for the prevention and treatment of disorders, such as hepatopathy[12] and nephropathy.[13] In this study, we estimated the antioxidative action of *Eriobotrya japonica* seed extract (ESE) with the ESR method, and the inhibitory activity on Fe^{3+}-ADP induced NADPH dependent lipid peroxidation was measured in rat liver microsomes. In addition, we combined them and examined the influence with the components of the ESE.

MATERIALS AND METHODS

Reagents β-Sitosterol and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nacalai Tesque Inc. Hypoxanthine (HPX), xanthine oxidase (XOD), 2-thiobarbituric acid (TBA), β-nicotinamide adenine dinucleotide phosphate reduced form (β-NADPH), sodium dodesylsulfate (SDS), DL-α-tocopherol and L-ascorbic acid were purchased from Wako Pure Chemical Industries, Ltd. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma and Dojindo Inc., respectively. The other reagents used were commercial guaranteed-grade reagents.

Preparation of Samples *Eriobotrya japonica* seeds were collected in Muroto and Suzaki, Kochi Prefecture, and dried well in the sun. *Eriobotrya japonica* seeds (1 kg) were crushed using a cooling apparatus-equipped blender (1000 rpm). After immersion in 2 l of 70% ethanol, the crushed seeds were stirred continuously using a stirrer (300 rpm, 7 d), and the supernatant was collected after 7 d. The collected 70% ethanol extracts were then combined and concentrated under reduced pressure until dry. The final yield of 70% ethanol extracts (ESE) was 108 g. ESE 108 g was extracted with 500 ml of Hex, 500 ml of EtOAc, 500 ml of *n*-BuOH, 500 ml of MeOH and 500 ml of H_2O, and the fractions were collected. The solvent of each extract was then removed under reduced pressure, and the resulting extracts were subsequently lyophilized to dryness. The final yield of Hex, EtOAc, *n*-BuOH, MeOH and H_2O extracts was 2.3, 2.4, 1.7, 10.2 and 89.8 g, respectively. The most antioxidation active strong H_2O fractions were sequentially purified using Diaion HP20 (0→100% MeOH), Sephadex LH20 and G50 (0→100% MeOH). The crude fractions were purified and the components were isolated. The structures of the isolated components were determined using JMN-NMR 400 MHz and HPLC (Instrument: HITACHI HPLC D-7000 series, column: Cosmosil 5C18-AR-II Waters 4.6×150 mm, mobile

* To whom correspondence should be addressed. e-mail: yokotaj@med.kochi-u.ac.jp
© 2006 Pharmaceutical Society of Japan

phase : A: 10 mM phosphate buffer (pH 2.6), B: acetonitrile (gradient: 0→15 min (Bconc. 0→30%), 15→18 min (Bconc. 30→70%), 18→30 min (Bconc. 70→10%)), flow rate: 1 ml/min, temperature: 40 °C, detector: Diode Array Detector (280, 320, 360 nm).

Polyphenols in ESE and the solvent fractions were measured according to the Folin–Denis method,[14] and the content was calculated as the (+)-catechin equivalent in the extract. Amino acid analysis was performed using by model L-8500A Hitachi high-speed amino acid analyzer. Also, β-sitosterol, fatty acids and amigdalin were confirmed using TLC according to the method of Nishioka *et al.*[12]

Antioxidative Activity. Measurement of Reactive Oxygen-Scavenging Action Using ESR[15] The ESR spectrum was measured using a JEOL JEX-RE3X ESR spectrophotometer (Nihon Densi Inc.). The measurement conditions are shown below. Power: 5 mW (DPPH), 8 mW ($\cdot O_2^-$), Field: 336.0±10 mT, Sweep time: 1 min, Modulation: 100 kHz 9.45 GHz, Time constant: 0.03 s, Receiver gain: 1×1000 (DPPH), 1.6×1000 (O_2^-). To evaluate the scavenging activity of each radical, the concentration–radical scavenging rate curve was prepared for each fraction and compound using the measurement method as below, and the concentration of 50% scavenging activity (IC_{50}). We used L-ascorbic acid and DL-α-tocopherol as the positive control.

DPPH Radical The stable free radical, DPPH, was dissolved in MeOH (100 μM). The DPPH solution 100 μl and sample solution 200 μl were mixed, and the DPPH radical was measured after 60 s. The sample was dissolved in mixture of acetone and 0.1 M phosphate buffer (pH 7.4) (1 : 1) and used. The concentration of the ESE, solvent fractions and the positive controls prepared were 1—10^{-4} mg/ml. The ESE components were prepared at 10 mg/ml (polyphenol 100 μg/ml). We used the spectrum that was observed when adding the solvent of the sample instead of the sample as a control. The signal intensity was investigated based on the ratio of the height of the external standard manganese signal and the height of the 3rd of 5 peaks of the DPPH radical.

O_2^--Scavenging Activity O_2^- radicals were generated with a hypoxanthine–xanthine oxidase system. All solutions were dissolved in 0.1 M phosphate buffer (pH 7.4). Samples that would not dissolve in 0.1 M phosphate buffer (pH 7.4) were dissolved in acetone. The concentration of acetone was prepared to be less than 1%. The concentration of ESE, solvent fractions and the positive controls were prepared at 20—0.1 mg/ml. ESE components were prepared at 10 mg/ml (polyphenol 100 μg/ml). 0.1 M phosphate buffer (pH 7.4) 90 μl, sample solution 50 μl, 2 mM HPX 50 μl, 5.5 mM DTPA 50 μl, 2.7 M DMPO 10 μl, and 0.27 units/ml XOD 50 μl were added in this order, and the DMPO-OO- spin adduct was measured using the ESR method 60 s after the addition of DMPO. The signal intensity was investigated based on the ratio of the height of the external standard manganese signal and the height of the 1st of 12 lines of DMPO-OO- spin adduct. The O_2^- scavenging activity was also used by measuring the SOD-like activity. The SOD-like activity is calculated from the relative peak height, utilizing that the signal intensity of DMPO-OO- decreases in the presence of SOD in a concentration-dependent manner. SOD is an antioxidative enzyme that scavenges O_2^- in the body. Production of the DMPO-OO adduct is inhibited by the scavenging of O_2^- by SOD in the ESR method. A calibration curve ($y=0.124x+0.13$, $r=0.992$) was prepared using standard SOD solutions (0.00—14.50 units/ml), and SOD equivalents were calculated.

Inhibition of Fe^{3+}-ADP Induced NADPH Dependent Lipid Peroxidation in Rat Liver Microsomes. Animals Male Wistar rats were purchased from NSC Japan. After purchase, the animals were maintained with free access to food and drinking water under constant temperature and humidity conditions for 1 week or longer before the experiment.

Preparation of Liver Microsomes Male Wistar rats were anesthetized with diethyl ether, and killed by exsanguination by cutting the axillary artery. A canula was inserted into the portal vein, and physiological saline was infused to flush out the blood. The liver was excised and washed with 0.25 M sucrose, cut into pieces and homogenized. The homogenate was centrifuged at 8000×g for 10 min at 4 °C. The supernatant was ultracentrifuged at 105000×g for 30 min at 4 °C, and the precipitate was collected. The protein in the precipitate was measured using the Lowry method,[16] and a microsome suspension was prepared (protein concentration: 20 mg/ml).

Anti-lipid Peroxidation Assay The reaction was measured according to the method reported by Kiso *et al.*[17] To the samples at various concentrations, 10 mM ADP, 2 mM β-NADPH, 20 mg protein/ml liver microsomes and 167 mM KCl-74.4 mM Tris–HCl buffer (pH 7.4) were added, and the solution was preincubated at 37 °C for 5 min. Subsequently, 0.1 mM $FeCl_3$ was added, followed by incubation at 37 °C for 20 min (final volume: 1 ml). After the reaction was stopped by cooling in ice, the peroxidized lipids were measured as purified malondialdehyde (MDA) according to the method reported by Ohkawa *et al.*[18] To the reaction solution, 8.1% SDS, 20% acetate–0.27 M HCl–NaOH buffer (pH 3.5) and 0.8% TBA were added. The mixture was heated in boiling water for 20 min. After the reaction was stopped by cooling in ice, *n*-BuOH : pyridine (15 : 1) was added, and the solution was vigorously mixed and centrifuged at 780×g for 10 min. The absorbance of the supernatant at 532 nm was measured, and the inhibition rate relative to the control was calculated.

RESULTS

Various Radical-Scavenging Activity of ESE and Solvent Fractions (ESR Method) Figure 1 shows the relationship between the concentrations of ESE and the solvent fractions and the DPPH and O_2^- radical-scavenging rates (%).

The various radical-scavenging rates varied among ESE and the solvent fractions, and the scavenging rate increased in a concentration-dependent manner for all of the radical species in ESE and the solvent fractions.

Table 1 shows the DPPH and O_2^- radical-scavenging activities (IC_{50} values) of ESE and the solvent fractions.

The IC_{50} value of ESE was 0.96×10^{-3} mg/ml to DPPH radical-scavenging activity. This showed a low value from DL-α-tocopherol (0.03 mg/ml), L-ascorbic acid (8.61×10^{-3} mg/ml), which was used as the positive control. The IC_{50} value of the DPPH radical-scavenging activity was the lowest in the H_2O fraction (0.62×10^{-3} mg/ml), showing a high scavenging activity. The scavenging activity decreased in the order of the MeOH (0.79×10^{-3} mg/ml), *n*-BuOH (0.94×

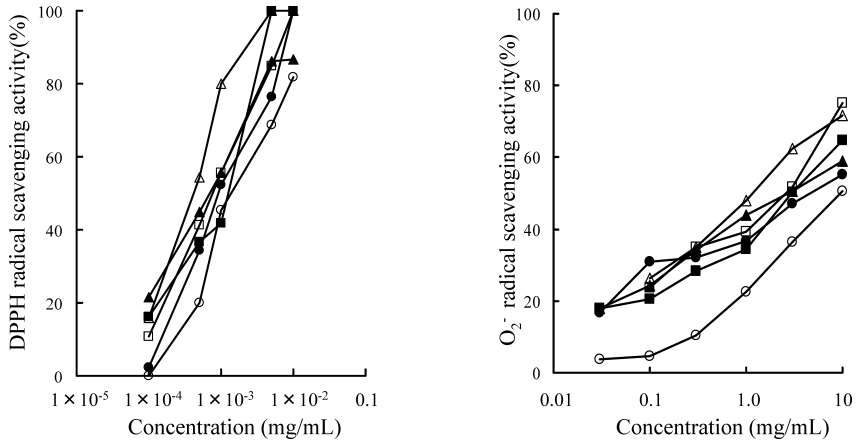

Fig. 1. Concentration Dependent Two Type of Radicals Scavenging Activity of *Eriobotrya japonica* Seed Extract and Several Solvents Fractions

●: ESE, ○: Hex fraction, ■: EtOAc fraction, □: *n*-BuOH fraction, ▲: MeOH fraction, △: water fraction. Each value is the mean of 6 observations.

Table 1. Free Radical Scavenging Activity of ESE and the Solvent Fractions in ESR

ESE, each fractions and positive control	IC_{50} (mg/ml)	
	DPPH radical	Super oxide
ESE	0.96×10^{-3}	2.88
Hex fraction	4.56×10^{-3}	13.44
EtOAc fraction	1.20×10^{-3}	2.85
n-BuOH fraction	0.94×10^{-3}	3.58
MeOH fraction	0.79×10^{-3}	2.00
Water fraction	0.62×10^{-3}	1.75
L-(+)-Ascorbic acid	8.61×10^{-3}	3.90
DL-α-Tocopherol	0.03	ND[a)]

Each value represents the mean of six tests. *a*) Not determined.

10^{-3} mg/ml), EtOAc (1.20×10^{-3} mg/ml), and Hex fractions (4.56×10^{-3} mg/ml).

The IC_{50} value of ESE was 2.88 mg/ml to the O_2^- radical-scavenging activity. This value was as high as L-ascorbic acid (3.90 mg/ml), which was used as the positive control. The IC_{50} value of the O_2^- radical-scavenging activity was the lowest in the H_2O fraction (1.75 mg/ml), showing high scavenging activity. As for the H_2O fraction, the O_2^- radical-scavenging activity was higher than ESE. The activity decreased in the order of MeOH (2.00 mg/ml), EtOAc (2.85 mg/ml), *n*-BuOH (3.58 mg/ml). The IC_{50} value of ESE was equivalent to SOD at 6.27 units/ml.

Lipid Peroxidation Inhibition Rates of ESE and the Solvent Fractions Figure 2 shows the relationship between the concentrations of ESE and the solvent fractions and the lipid peroxidation inhibition rates (%). The lipid peroxidation inhibition rate varied among ESE and the solvent fractions, and increased in a concentration-dependent manner in ESE and the solvent fractions. The lipid peroxidation inhibition rate was not markedly different among the solvent fractions, unlike the radical scavenging activity.

Table 2 shows the lipid peroxidation inhibition activity (IC_{50} values) of ESE and the solvent fractions. The lipid peroxidation inhibition activity (IC_{50} value) was the lowest in the H_2O fraction (10.08 μg/ml), showing a high inhibitory activity. Unlike the radical scavenging activity, the inhibitory activity was also high in the low-polar Hex and EtOAc fractions (12.34 and 15.62 μg/ml, respectively). The value of DL-α-tocopherol, which was the positive control, was 40.21 μg/ml. For lipid peroxidation, ESE and the solvent fractions were stronger than DL-α-tocopherol.

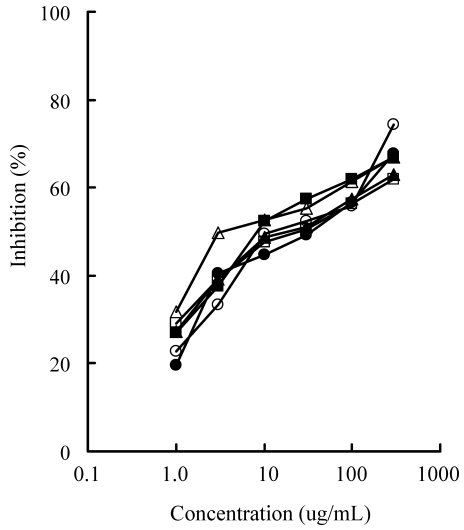

Fig. 2. Effect on Fe^{3+}-ADP Induced NADPH Dependent Lipid Peroxidation in Rat Liver Microsomes

●: ESE, ○: Hex fraction, ■: EtOAc fraction, □: *n*-BuOH fraction, ▲: MeOH fraction, △: water fraction. Each value is the mean of 6 observations.

Table 2. Anti-lipid Peroxidation Activity of *Eriobotrya japonica in Vitro*

ESE, each fractions and positive control	IC_{50} (μg/ml)
ESE	30.35
Hex fraction	12.34
EtOAc fraction	15.62
n-BuOH fraction	28.64
MeOH fraction	22.34
Water fraction	10.08
L-(+)-Ascorbic acid	ND[a)]
DL-α-Tocopherol	40.24

Each value represents the mean of six tests. *a*) Not determined.

Investigation of ESE Components Because the antioxidative activity of ESE and the solvent fractions are different, we examined the components of ESE to clarify the different

Table 3. The Amounts of Containing Polyphenols in ESE

ESE and each fractions	Polyphenol (content %)
ESE	0.54
Hex fraction	0.00
EtOAc fraction	0.22
n-BuOH fraction	0.23
MeOH fraction	0.36
H_2O fraction	0.56

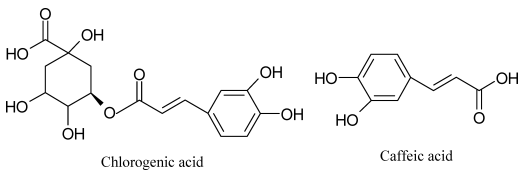

Fig. 3 Chemical Structures of Polyphenols from *Eriobotrya japonica* Seed Extract

Table 4. The Containing Compounds of *Eriobotrya japonica* (Amino Acid)

Essential amino acids		Others	
Isoleucine	(Ile)	Alanine	(Ala)
Leucine	(Leu)	Arginine	(Arg)
Lysine	(Lys)	Aspartic acid	(Asp)
Phenylalanine	(Phe)	Glutamic acid	(Glu)
Threonine	(Thr)	Glycine	(Gly)
Tryptophan	(Trp)	Proline	(Pro)
Valine	(Val)	Serine	(Ser)
		Tyrosine	(Tyr)

antioxidations. Table 3 shows the polyphenol content (%) in ESE and the solvent fractions. The polyphenol content (%) varied among the solvent fractions. The content per weight of fraction was 0.56% in the H_2O fraction, which is the highest and was equivalent to ESE. The content decreased in the order of MeOH, *n*-BuOH and EtOAc fractions. No polyphenol was contained in the Hex fraction.

We performed detailed examination about a component of the H_2O fraction whose antioxidation activity was the highest. The structures of 2 polyphenols were determined. Figure 3 shows the structural formula. ESE contained 2.1 mg caffeic acid and 8.7 mg chlorogenic acid, which are phenyl propanoids, per 100 g. Table 4 shows the results of amino acid analysis of ESE. Fifteen amino acids were detected in ESE, and 7 of these were essential amino acids.

Free Radical Scavenging Activities and the Anti-lipid Peroxidation of ESE Components The anti-lipid peroxidation and free radical scavenging activities of ESE components were tested. The activity was lower in all of the compounds, compared to the above polyphenols, and comparison by calculation of the IC_{50} value was difficult. Thus, the scavenging rates (%) at a concentration of 10 mg/ml were presented. The polyphenol showed a value in 100 μg/ml. As for the polyphenols, having the highest antioxidative action was suggested in the components. The antioxidative activity of fatty acids was not shown. However, β-sitosterol exhibited a higher lipid peroxidation inhibition rate.

Table 5. Radical Scavenging Activity of ESE Contents

Pure compounds	Radical scavenging activity (%)		
	DPPH	Super oxide	Anti-lipid peroxidation
Caffeic acid[a)]	85.9	80.1	76.5
Chlorogenic acid[a)]	88.5	79.3	72.8
β-Sitosterol	10.7	<1	76.3
Linolic acid	25.3	12.6	15.8
Linoleic acid	<1	4.5	10.2
Amigdaline	<1	10.3	<1
Benzaldehyde	<1	<1	<1
Benzoic acid	<1	<1	<1
Mandelonitril	7.0	23.7	6.8

Each value represents the mean of six tests. Concentration of each compound was 10 mg/ml. *a*) Each value represents the mean of six tests. Concentration of each compound was 100 μg/ml.

DISCUSSION

Various radicals are produced in oxidative reactions in the body, and exert useful actions for the body in the immune system.[19] However, various excessively produced radicals react with biological components, such as DNA, proteins and phospholipids, and induce oxidative disorders, leading to heart disease, diabetes and cancer.[20] Antioxidative substances are expected to be effective for such disorders.[21,22]

In the previous report,[13] we clarified that ESE was useful for the improvement of nephropathy in rats with adriamycin-induced nephropathy, and the action was not indirectly exerted through antioxidative enzymes, but was due to the direct antioxidative action of ESE components absorbed in the body.

In this study, we clarified the components of ESE and investigated their antioxidative action. Regarding the various radical scavenging activities and lipid peroxidation inhibition activity of ESE, a high antioxidative action was detected in the high-polar solvent fractions when ESE was fractionated with solvents with different polarities. This finding clarified the involvement of the high-polar solvent fractions in the antioxidative action of ESE.

The components were investigated in each fraction, and the high-polar solvent fractions contained abundant polyphenols. These polyphenols were isolated, and the structures were determined. As a result, caffeic acid and chlorogenic acid were identified. These components exhibited high antioxidative action, clarifying that these polyphenols are closely involved in the exertion of the antioxidative action of ESE. In the low-polar Hex and EtOAc fractions containing no polyphenols, the radical scavenging activity was low, but the lipid peroxidation inhibition activity was high, suggesting the involvement of other substances. The radical scavenging activity and the lipid peroxidation inhibition activity of the other substances were lower than those of the polyphenols. However, as Nishioka *et al.* reported,[13] it was suggested that β-sitosterol, which inhibits active oxygen produced by neutrophils, exerts its antioxidative action through a preventive action of β-sitosterol, such as stabilization of the cell membrane. Amino acids are components of an antioxidative enzyme in the body, catalase and glutathione, suggesting that the antioxidative action of amino acids is not exerted by

amino acids themselves, and various physiological functions, such as antioxidative action, are exhibited through absorption into the body.[23,24)]

Based on the above information, caffeic acid derivatives and polyphenols that capture $\cdot OOH$, $\cdot OH$ and $\cdot O_2^-$ as radical scavengers, and β-sitosterol, which exerts a preventive action by inhibiting the excess production of active oxygen by various cells, may be involved in the antioxidative action of ESE in a complex manner.

Multiple components of ESE may act additively or synergistically, and have a direct antioxidative action and biological regulatory function, which is useful for the prevention and treatment of various disorders.

Further investigation of components, and the effects on other active oxygen species, such as NO and related disorders, is necessary.

REFERENCES

1) Aust S. D., Chignell C. F., Bray T. M., Kalyanaraman B., Mason R. P., *Toxicol. Appl. Pharmacol.*, **120**, 168—178 (1993).
2) Stohs S. J., *J. Basic Clin. Physiol. Pharmacol.*, **6**, 205—228 (1995).
3) Block G., *Nutr. Rev.*, **50**, 207—213 (1992).
4) Noda Y., Anzai K., Mori A., Kohno M., Shinmei M., Packer L., *Biochem. Mol. Biol. Int.*, **42**, 35—44 (1997).
5) De Tommasi N., De Simone F., Cirino G., Cicala C., Pizza C., *Planta Med.*, **57**, 414—416 (1991).
6) Roman-Ramos R., Flores-Saenz J. L., Partida-Hernandez G., Lara-Lemus A., *Arch. Invest. Med.* (Mex), **22**, 87—93 (1991).
7) Alippi A. M., Alippi H. E., *Rev. Argent. Microbiol.*, **22**, 155—158 (1990).
8) Young H. S., Chung H. Y., Lee C. K., Park K. Y., Yokozawa T., Oura H., *Biol. Pharm. Bull.*, **17**, 990—992 (1994).
9) De Tommasi N., De Simone F., Pizza C., Mahmood N., Moore P. S., Conti C., Orsi N., Stein M. L., *J. Nat. Prod.*, **55**, 1067—1073 (1992).
10) Ito H., Kobayashi E., Takamatsu Y., Li S. H., Hanano T., Sakagami H., Kusama K., Saotoh K., Sugita D., Shimura S., Itoh Y., Yoshida T., *Chem. Pharm. Bull.*, **48**, 687—693 (2000).
11) Gray D. O., *Phytochemistry*, **11**, 745—751 (1972).
12) Nishioka Y., Yoshioka S., Kusunose M., Cui T., Hamada A., Ono M., Miyamura M., Kyotani S., *Biol. Pharm. Bull.*, **25**, 1053—1057 (2002).
13) Hamada A., Yoshioka S., Takuma D., Yokota J., Cui T., Kusunose M., Miyamura M., Kyotani S., Nishioka Y., *Biol. Pharm. Bull.*, **27**, 1961—1964 (2004).
14) Appel H. M., Govenor H. L., D'Ascenzo M., Siska E., Schultz J. C., *J. Chem. Ecol.*, **27**, 761—778 (2001).
15) Fushitani S., Tsuchiya K., Minakuchi K., Takasugi M., Murakami K., *Yakugaku Zasshi*, **114**, 388—394 (1994).
16) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
17) Kiso Y., Tohkin M., Hikino H., Hattori M., Sakamoto T., Namba T., *Planta Med.*, **50**, 298—302 (1984).
18) Ohkawa H., Ohnishi N., Yagi K., *Anal. Biochem.*, **95**, 351—358 (1979).
19) Vuillaume M., *Mutation Res.*, **186**, 43—72 (1987).
20) Slater T. F., *Biochem. J.*, **222**, 1—15 (1984).
21) Halliwell B., Gutteridge J. M. C., *Lancet*, **23**, 1396—1397 (1984).
22) Hochstein P., Atallah A. S., *Mutation Res.*, **202**, 363—375 (1988).
23) Goldberg A. L., *Federation Proc.*, **37**, 2301—2307 (1978).
24) Okada A., Mori S., Totsuka M., Okamoto K., Usui S., Fujita H., Itakura T., Mizote H., *J. Parenteral Enteral Nutrition*, **12**, 332—337 (1988).