Synthesis and *in Vitro* Antioxidant Activity of Glycyrrhetinic Acid Derivatives Tested with the Cytochrome P450/NADPH System

Mourboul Ablise,[a,b] Brigitte Leininger-Muller,[a] Choi Dal Wong,[a] Gérard Siest,*[,a] Vincent Loppinet,[a] and Sophie Visvikis[a]

[a] *INSERM Unité 525, Centre du Médicament, Faculté de Pharmacie, Université Henri Poincaré Nancy 1; 54000 Nancy, France: and* [b] *College of Pharmacy, Xinjiang Medical University; 830054-Urumqi, Xinjiang, P. R. China.*
Received May 20, 2004; accepted July 21, 2004

Five glycyrrhetinic acid (Ib) derivatives have been synthesized to try to improve the antioxidant activity. Their *in vitro* antioxidant activities were studied using a cytochrome P450/NADPH reductase system from rat liver microsomes. The generation of microsomal free radicals was followed by oxidation of the DCFH-DA probe, while evaluating the capacity to inhibit reactive oxygen species (ROS) formation. Two hydroxylated derivatives, 18β-olean-12-ene-3β,11α,30-triol (II) and 18β-olean-12-ene-3β,11β,30-triol (IV), exhibited strong antioxidant activities. At a concentration of 1.0 mg/ml, these derivatives inhibited ROS formation by 50% and 51%, respectively. Moreover, two homo- and heterocyclic diene derivatives, 18β-olean-11,13(18)-diene-3β,30-diol (III) and 18β-olean-9(11),12-diene-3β,30-diol (V), were also effective in ROS-scavenging activity (inhibition of 41% and 44% of ROS activity, respectively). In the same conditions, the lead compound (Ib) and the reference vitamin E inhibited ROS activity by 31% and 32%, respectively. Our results suggest that the chemical reduction of the 11-keto and 30-carboxyl groups into hydroxyl function (example, II, IV) can increase the antioxidant activity of Ib significantly. In view of these results, our study represents a further approach to the development of potential therapeutic agents from Ib derivatives for use in pathologic events in which, free radical damage could be involved.

Key words glycyrrhetinic acid derivative; antioxidant activity; cytochrome P450/NADPH reductase

The involvement of free radicals in the pathology of human diseases such as atherosclerosis, cardiovascular diseases, and diabetes, has been recognized by a number of authors.[1—3] Protective therapeutic intervention might include natural or synthetic pharmacologic agents with antioxidant activity.[4] Therapeutic agents with multiple mechanisms of protective action, including antioxidant prophylactic properties, may become valuable in attempts to minimize tissue injury in human disease. This may be helpful in pharmaceutical R&D strategies.[4]

Due to multiple protective actions of a triterpenoid from licorice,[5] we have developed some semisynthetic derivatives of glycyrrhetinic acid (**Ib**), the aglycone of glycyrrhizin (**Ia**) which is a major triterpenoic saponin of licorice, and examined their antioxidant capacity. A derivative of licorice root (*Glycyrrhiza glabra* L.) and its main water-soluble constituent glycyrrhizin (**Ia**), a pentacyclic triterpene derivative of the *beta*-amyrin type (oleanane), has been widely used as an antidote, a food additive, and a folk medicine for generations in Asia and Europe.[6] **Ia** can be hydrolyzed into active aglycone, 18-*beta*-glycyrrhetinic acid (**Ib**). Both **Ia** and **Ib** have been shown to have several beneficial pharmacologic activities, such as antiinflammatory activity,[7] antiulcerative effect,[8] antiviral activity,[9] and antihepatitis effect.[10]

Recently, Zakirov and Abdullaev[11] have demonstrated the effective antioxidant and hypolipidemic properties of ammonium salt of **Ib**. It increases superoxide dismutase (SOD) activity and decreases the concentration of malondialdehyde (MDA) and lowers the cholesterol and triglyceride levels by 51% and 71%, respectively, at a concentration of 25 mg/kg in atherosclerotic and hypercholesterolemic rabbits. As a result, it effectively inhibits the progression of atherosclerotic lesion.[11]

Hye *et al.*[12] reported the protective effects of **Ib** against carbon tetrachloride-induced hepatotoxicity and the possible mechanisms involved in this protection. Shibata *et al.*[13] synthesized some new derivatives of **Ib** which suppressed its pseudoaldosteronism side effects, and Kiso *et al.*[14] demonstrated the effective antiinflammatory properties of those derivatives. Nevertheless, the antioxidant effects of these derivatives have not been systematically reported.

The multiple beneficial effects such as antioxidant, hypolipidemic, anti-inflammatory, and antiatherosclerosis effects of **Ib** point to the possibility of developing new active cardiovascular agents from this structural family. We structurally modified **Ib** and prepared five synthetic derivatives to improve the antioxidant activity. We performed a comparative study of their *in vitro* antioxidant properties in a microsomal free radical-generating system and found significant results with increased antioxidant activity for some. In addition, we carried out a computerized hypolipidemic pharmacophore identification of these derivatives with receptor mapping methods and propose a hypothesis to explain their possible hypolipidemic mechanism of action.[15]

Chemistry All derivatives were prepared by the methods previously described[13] (Fig. 1). Many preparative procedures have also been optimized. Other chemicals were obtained from the usual commercial sources. **Ia** was purified from licorice root and hydrolyzed into **Ib** (step a). We reduced **Ib** into the two stereoisomers **II** and **IV** (step b) with an optimized procedure. Dehydration of triol (**IV**) into diene (steps c-$_1$, c-$_2$) was conducted to obtain **V** and **III** in a 3 : 2 ratio. The product **VI** was obtained by a reduction of the mixture **II**+**IV** by catalytic hydrogenation in Pd–C at ambient temperature (step c-$_3$) and in absolute ethanol presaturated with $NaHCO_3$, while rigorously controlling hydrogen

* To whom correspondence should be addressed. e-mail: Gerard.Siest@pharma.uhp-nancy.fr

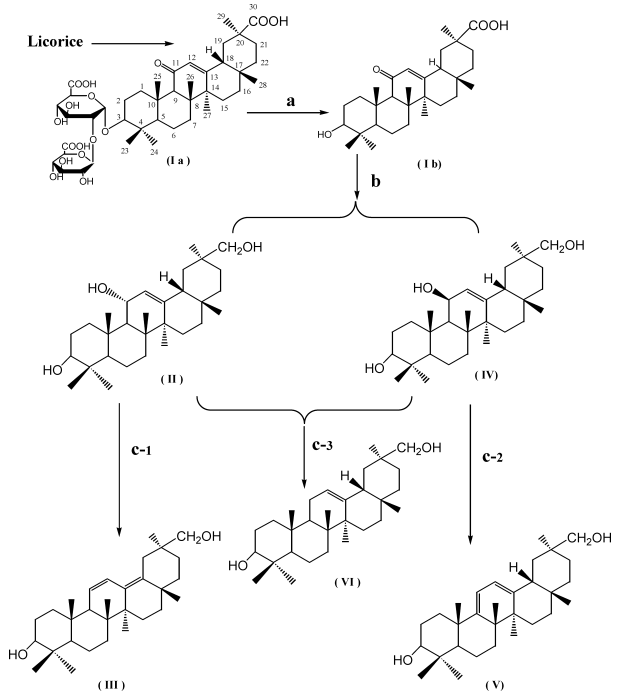

Fig. 1. Synthesis Pathway of Glycyrrhetinic Acid Derivatives

Ia, glycyrrhizin; **Ib**, 3β-hydroxy-11-oxo-18β-olean-12-en-30-oic acid; **II**, 18β-olean-12-ene-3β,11α,30-triol; **III**, 18β-olean-11,13(18)-diene-3β,30-diol; **IV**, 18β-olean-12-ene-3β,11β,30-triol; **V**, 18β-olean-9(11),12-diene-3β,30-diol; **VI**, 18β-olean-12-ene-3β,30-diol.

consumption.

Biochemical The *in vitro* antioxidant activities of these derivatives were tested with a major free radical-generating system, cytochrome P450/NADPH reductase, from rat liver microsomes. The typical antioxidant vitamin E served as a positive control.

The rate of reactive oxygen species (ROS) generation was quantified using the fluorescent probe precursor, 2,7-dichlorofluorescein diacetate (DCFH-DA), a probe that has been utilized extensively for the measurement of microsmal ROS production.[16,17] This assay has been shown to detect the fluorescence associated with the oxidation of DCFH by several reactive intermediates, including superoxide anions, H_2O_2, and hydroxyl radicals in microsomes.[16] Microsomes (rate, 0.5 mg/ml) were preincubated for 15 min with 4 μM of DCFH-DA for deesterification by endogenous esterase in phosphate buffer 50 mM, pH 7.4, at 37 °C. Then the mixture was incubated for an additional 30 min in the presence or absence of NADPH 0.5 mM and a sample (20 μl) using the method described by Choi *et al.*[18] At the end of incubation, fluorescent DCF formation was monitored using an FL 600 Microplate Fluorescence Reader (Bio-Tek, Highland Park, U.S.A.) at an excitation and emission wavelength of 485 and 530 nm, respectively. The rate of ROS generation was linear over the incubation period. A standard curve using DCF 0.01—0.10 μM was prepared and the results are expressed as picomoles of DCF formed per minute per milligram of protein.

The experiment was conducted at least three times. Statistical analysis was performed using one-way ANOVA (SAS software, SAS Institute Inc., NC). A value of $p<0.05$ was considered statistically significant. All biochemical results are reported in Figs. 2 and 3.

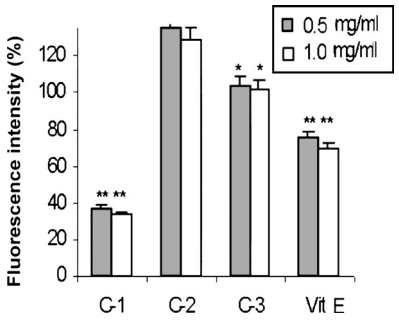

Fig. 2. NADPH-Initiated Microsomal Oxidation of DCFH-DA and Its Modification in the Presence of Vitamin E

The reaction mixture was composed of the microsome suspension (0.5 mg/ml), NADPH-generating system, DCFH-DA and vitamin E. After incubation at 37 °C for 30 min, microsomal oxidation was measured by the methods described. Values are the mean±S.D. of three individual determinations. Asterisks indicate a significant difference from the NADPH control (one-way ANOVA test, SAS software) $*p<0.05$, $**p<0.01$. Control 1 (C1), microsome alone; control 2 (C2), microsome+NADPH; control 3 (C3), microsome+NADPH+DMSO/ethanol; Vit E, vitamine E+NADPH+DMSO/ethanol.

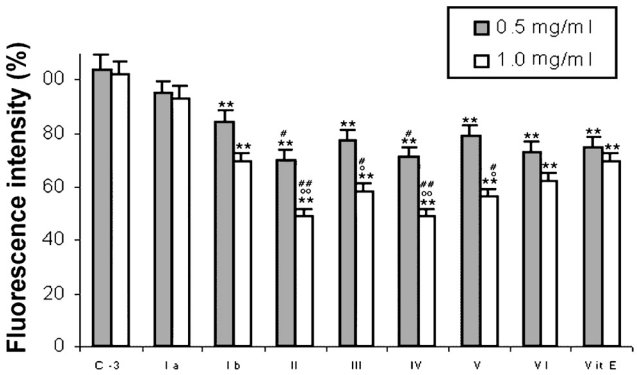

Fig. 3. NADPH-Initiated Microsomal Oxidation of DCFH-DA and Its Modification in the Presence of Glycyrrhetinic Acid Derivatives (**Ia**—**VI**) and Vitamin E (Vit E)

The reaction mixture was composed of the microsome suspension (0.5 mg/ml), NADPH-generating system, DCFH-DA, and the derivatives. After incubation at 37 °C for 30 min, microsomal oxidation was measured. Values are the mean±S.D. of three individual determinations. Asterisks indicate a significant difference from the NADPH control. C3, microsome+NADPH+DMSO/ethanol. * Significant difference *vs.* C3 ($*p<0.05$, $**p<0.01$); ° significant difference *vs.* vitamin E ($\circ p<0.05$, $\circ\circ p<0.01$); # significant difference *vs.* **Ib** ($\#p<0.05$, $\#\#p<0.01$) (one-way ANOVA test with SAS software).

Results and Discussion

Chemistry We applied a destruction method of aluminum salt with a calculated quantity of NaOH in reaction step b to minimize the rate of the second reaction (dehydratation of triols) and to facilitate the separation of product **II** from **IV** (the two stereoisomers) and from the byproduct aluminum salt. With this method, we achieved good separation of isomer **IV** with high purity with a simple crystallization method in chloroform and then in THF/AcOH. The isomer **II** was purified using the silica gel chromatographic method.

Dehydration of the triol **IV** into a diene (reaction step C-2) allows us to obtain a mixture of **V** and **III** in a 3 : 2 ratio. **III** was purified by recrystallization in absolute ethanol with $NaHCO_3$ under pressure, and **V** was purified by recristallization in chloroform. The separation process was performed using TLC and HPLC methods.

The product **VI** was obtained by reduction of a mixture of **II**+**IV** by catalytic hydrogenation in Pd-C, at ambient temperature and in absolute ethanol presaturated with $NaHCO_3$. It was purified by recrystallisation in ethyl acetate with a yield of 90%.

Biochemistry Samples were subsequently added to the biological oxidative system at a concentration of 0.50 and 1.00 mg/ml. The addition of NADPH to rat liver microsomes initiated DCFH-DA oxidation. This oxidation was elevated more than four-fold in the presence of 0.5 mM of NADPH (428±46 pmol/min/mg protein). At first, the addition of vitamin E (a known ROS scavenger) at an concentration of 1.0 mg/ml to the oxidation system resulted a diminution of DCFH-DA oxidation by 32%, as shown in Fig. 2. Second, the addition of 25 μM of DPI (an inhibitor of flavin oxidoreductase such as NADPH-cytochrome P450 reductase)[19] to the system resulted in suppression of NADPH-dependent electron transfer, and the oxidation of DCFH-DA was inhibited by 85%. These results demonstrate that ROS scavengers and inhibitors of microsomal enzyme can prevent the NADPH-dependent oxidation of DCFH-DA. We used this successful oxidation system to evaluate the NADPH-dependent ROS inhibition properties of our derivatives. We subsequently observed the inhibitory properties of NADPH-generated ROS production by the prepared derivatives. We found that the two hydroxylated derivatives **II** and **IV** have the most potent antioxidant activity. At a concentration of 1.0 mg/ml, they inhibited ROS formation by 50% and 51%, respectively, in the microsomal system. Moreover, two homo- and heterocyclic diene derivatives **III** and **V** inhibited ROS activity by 41% and 44%, respectively. Under the same conditions, the lead compound **Ib** inhibited ROS activity by 31%. The ROS inhibitory capacity of these derivatives increased with increasing concentration. We found that the saponin **Ia** had less antioxidant activity. All the results are shown in Fig. 3. Furthermore, we observed that the addition of an ethanol and DMSO mixture used to solubilize the products decreased the DCFH-DA oxidation levels relative to the control 2.

In the present study, we examined the initial structure–activity relationships of these derivatives by comparing the antioxidant actions monitored by the inhibition of microsomal ROS formation. Compounds **II** and **IV** showed the most intensive activity. The activity of all derivatives was in the decreasing order **IV**≥**II**>**V**>**III**>**VI**>**Ib**>**Ia**. From these results, it can be concluded that chemical reduction of 11-keto and 30-carboxyl functions into alcohol groups can increase the free radical-scavenging activity of the lead compound (**Ib**). However, it is also known that dehydratation of the 11-hydroxyl group gives the homo- and heterocyclic diene derivatives with relatively potent antioxidant activity.

Our findings indicate that the antioxidant property of some synthetic derivatives of **Ib** could represent potential therapeutic activity with multiple protective mechanisms against free radical damage, such as antioxidant, anti-inflammatory, and hypolipidemic properties.

Experimental

Chemistry Melting points were determined on a Kofler apparatus. Reaction courses were routinely monitored using thin-layer chromatography (TLC) on silica gel 60 F254 (Merck) with detection under a 254-nm UV lamp. ^{1}H-NMR spectra were determined in $CDCl_3$ solution with a Bruker DRX-400 (400 MHz) spectrometer and chemical shifts are presented in ppm from internal tetramethylsilane as a standard. Mass spectroanalyses were performed with a quadrupolar spectrometer VG Platform II. HPLC analysis was performed on a Merck L-3000/diode array with a Lichrospher-100 diol column (5 μm, 25 cm). All compounds showed a purity higher than 99% following analytical HPLC. Other analytical parameters including UV (UV Krontron) and IR (with KBr) spectra were also examined.

18β-Glycyrrhetinic Acid. 3β-Hydroxy-11-oxo-18β-olean-12-en-30-oic (Ib) Glycyrrhizin (**Ia**), a diglucuronide saponin of licorice root (*G. glabra*), colorless needless, mp 335 °C, was hydrolyzed to prepare **Ib**, which was used as a starting compound for chemical modification. **Ib**: Colorless needles, yield 70%, mp 296 °C (EtOH). ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.86 (3H, s, C-24 H_3), 1.03 (3H, s, C-26 H_3), 1.15 (3H, s, C-23 H_3), 1.16 (3H, s, C-25 H_3), 1.25 (3H, s, C-29 H_3), 1.39 (3H, s, C-27 H_3), 2.37 (1H, s, C-9 H), 2.81(2H, dt/3.20, 13.6, C-1 H_2), 3.26 (1H, dd/5.80, 10.80, C-3 H), 5.73 (1H, s, C-12 H). IR (KBr) cm^{-1} ν: 3440 (OH), 3046 (C sp^2), 2943 (C sp^3), 1666 (C=O, Cα=Cβ), 1018 (C-3 OH); δ: 1446 (CH_2, α CH_3), 1375 (s CH_3).

18β-Olean-12-ene-3β,11α,30-triol (II) Colorless needles, yield 1%, mp 222—225 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.86 (3H, s, C-24 H_3), 0.93 (3H, s, C-26 H_3), 1.02 (3H, s, C-25 H_3), 1.03 (3H, s, C-29 H_3), 1.08 (3H, s, C-23 H_3), 1.28 (3H, s, C-27 H_3), 3.26 (1H, dd/5.80, 10.80, C-3 H), 3.55 (2H, AB/-10.96, C-30 H_2), 4.21 (1H or 2H, dd/3.62, 8.16, C-11 H), 5.26 (1H, dd/3.58, C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3368 (OH), 3061 (C sp^2), 2924 (C sp^3), 1655 (C=C), 1029 (C-3 or 11 OH), δ: 1461 (CH_2, α CH_3), 1375 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3706. TLC *Rf*=0.19 (ethyl acetate–*n*-hexane, 70 : 30).

18β-Olean-11,13(18)-diene-3β,30-diol (III) Colorless needles, yield 31%, mp 228—230 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.73 (3H, s, C-26 H_3), 0.87 (3H, s, C-25 H_3), 0.87 (3H, s, C-28 H_3), 0.92 (3H, s, C-24 H_3), 0.98 (3H, s, C-29 H_3), 1.07 (3H, s, C-23 H_3), 1.09 (3H, s, C-27 H_3), 3.26 (1H, dd/4.95, 11.28), 3.38 (2H, s, C-30 H_2), 5.56 (1H, d large/10.72, C-12 H), 6.39 (1H or 2H, dd/2.66, 10.36, C-11 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3353 (OH), 3040 (C sp^2), 2856—2974 (C sp^3), 1637 (C=C), 1022 (C-3 or 11 OH), 926 (C-30 OH), δ: 1478 (CH_2, α CH_3), 1360 (s CH_3). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found: M$^+$ 440.3658. TLC *Rf*=0.43 (CH_2Cl_2–MeOH, 96 : 4). HPLC t_R=12.8 min.

18β-Olean-12-ene-3β,11β,30-triol (IV) Colorless needles, yield 77%, mp 238—239 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.85 (3H, s, C-28 H_3), 0.89 (3H, s, C-24 H_3), 0.93 (3H, s, C-26 H_3), 1.02 (3H, s, C-25 H_3), 1.13 (3H, s, C-23 H_3), 1.26 (3H, s, C-29 H_3), 1.42 (3H, s, C-27 H_3), 3.26 (1H, dd/5.41, 10.59, C-3 H), 3.55 (2H, AB/-10.77, C-30 H_2), 4.33 (1H or 2H, s large pic, C-11 H), 5.33 (1H, d/3.94, C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3412 (OH), 2927 (C sp^3), 1634 (C=C), 1022 (C-3 or 11 OH), δ: 1456 (CH_2, α CH_3), 1382 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_3$: M$^+$ 458.3760. Found: M$^+$ 458.3728. TLC *Rf*=0.31 (ethyl acetate–*n*-hexane, 70 : 30).

18β-Olean-9(11),12-diene-3β,30-diol (V) Colorless needles, yield 63%, mp 236 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.83 (3H, s, C-28 H_3), 0.91 (3H, s, C-26 H_3), 0.92 (3H, s, C-24 H_3), 1.03 (3H, s, C-27 H_3), 1.05 (3H, s, C-25 H_3), 1.15 (3H, s, C-23 H_3), 1.21 (3H, s, C-29 H_3), 3.26 (1H, t/5.87, C-3 H), 3.57 (2H, AB/-10.64, C-30 H_2), 5.56 (1H or 2H, dd/5.96, 25.22, C-11 H and C-12 H). Not detected: C-1 H_2, C-9 H_1. IR (KBr) cm^{-1} ν: 3390 (OH), 3038 (C sp^2), 2978—2853 (C sp^3), 1628 (C=C), 1029 (C-3 OH), 985 (C-30 OH), δ: 1449 (CH_2, α CH_3), 1360 (s CH_3). MS *m/z* Calcd for $C_{30}H_{48}O_2$: M$^+$ 440.3654. Found: M$^+$ 440.3663. TLC *Rf*=0.43 (CH_2Cl_2–MeOH, 96 : 4). HPLC t_R=12.2 min.

11-Deoxo-glycyrrhetol (18β-Olean-12-ene-3β,30-diol) (VI) Colorless needles, yield 90%, mp 250—254 °C. ^{1}H-NMR ($CDCl_3$) δ ppm: 0.81 (3H, s, C-28 H_3), 0.85 (3H, s, C-26 H_3), 0.92 (3H, s, C-25 H_3), 0.96 (3H, s, C-24 H_3), 0.98 (3H, s, C-29 H_3), 1.02 (3H, s, C-23 H_3), 1.17 (3H, s, C-27 H_3), 3.25 (1H, dd/4.41, 11.93, C-3 H), 3.54 (2H, AB/-10.82, C-30 H_2), 5.21 (1H, t/3.21, C-12 H). Not detected: C-1 H_2, C-9 H_1, C-11 $H_{1\ or\ 2}$. IR (KBr) cm^{-1} ν: 3268 (OH), 3081 (C sp^2), 2971—2860 (C sp^3), 1691 (C=C), 1022 (C-3 OH), 926 (C-30 OH), δ: 1471—1390 (s CH_3). MS *m/z* Calcd for $C_{30}H_{50}O_2$: M$^+$ 442.3811. Found: M$^+$ 442.3842. TLC *Rf*=0.23 (CH_2Cl_2–MeOH, 96 : 4).

Biochemistry Animals and Microsome Preparation: Male Sprague-Dawley rats (150—180 g, Iffa-Credo, France) were housed in a controlled environment room with a 12-h light/dark photoperiod. The rats were fasted overnight prior to killing. Liver microsomes were prepared by differential centrifugation as previously described.[20] Protein concentrations were determined using the method of Lowry *et al.*[21] with bovine serum albumin as a standard.

Acknowledgment The authors thank Dr. J. C. Ziegler for providing chemical synthesis experience.

References

1) Leborgne L., Maziere J. C., Maziere C., Andrejak M., *Arch. Mal. Cœur. Vaiss.*, **95**, 805—814 (2002).
2) Folts J. D., *Adv. Exp. Med. Biol.*, **505**, 95—111 (2002).
3) Petrovsky N., Silva D., Schatz D. A., *Paediatr. Drugs*, **5**, 575—582 (2003).
4) Aruoma O. I., *Free Rad. Biol. Med.*, **20**, 675—705 (1996).
5) Baltina L. A., *Curr. Med. Chem.*, **10**, 155—171 (2003).
6) Shibata S., *Yakugaku Zasshi*, **120**, 849—862 (2000).
7) Inoue H., Nagata N., Shibata S., Koshihara Y., *Jpn. J. Pharmacol.*, **71**, 281—289 (1996).
8) Farina C., Pinza M., Pifferi G., *Farmaco.*, **53**, 22—32 (1998).
9) Badam L., *J. Commun. Dis.*, **29**, 91—99 (1997).
10) Arase Y., Ikeda K., Murashima N., Chayama K., Tsubota A., Koida I., Suzuki Y., Saitoh S., Kobayashi M., Kumada H., *Cancer*, **79**, 1494—1500 (1997).
11) Zakirov U. B., Abdullaev A. K., *Eksp. Klin. Farmakol.*, **59**, 53—55 (1996).
12) Hye G. J., Sung J. P., Ae R. M., Young C. C., Shin K. K., Hyo K. C., *Pharmacol. Res.*, **46**, 221—227 (2002).
13) Shibata S., Takahashi K., Yano S., Harada M., Saito H., Tamura Y., Kumagai A., Hirabayashi K., Yamamoto M., Nagata N., *Chem. Pharm. Bull.*, **35**, 1910—1918 (1987).
14) Kiso Y., Tohkin M., Hikino H., Hattori M., Sakamoto T., Namba T., *Planta Med.*, **50**, 298—302 (1984).
15) Ablise M., Cartier A., Siest G., Visvikis S., Loppinet V., *Mini Rev. Med. Chem.*, **2**, 97—102 (2002).
16) Puntarulo S., Cederbaum A. I., *Free Rad. Biol. Med.*, **24**, 1324—1330 (1998).
17) Serron S. C., Dwivedi N., Backes W. L., *Toxicol. Appl. Pharmacol.*, **164**, 305—311 (2000).
18) Choi D. W., Leininger-Muller B., Young C. K., Leroy P., Siest G., Wellman M., *Free Rad. Res.*, **36**, 893—903 (2002).
19) McGuire J. J., Anderson D. J., McDonald B. J., Narayanasami R., Benett B. M., *Biochem. Pharmacol.*, **56**, 881—893 (1998).
20) Extrom G., Ingelman-Sundberg M., *Biochem. Pharmacol.*, **38**, 1313—1319 (1989).
21) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).