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Efficiency and structure-activity relationship of the antioxidant action of resveratrol and its analogs

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Resveratrol and its analogs, six other polyhydroxystilbenes, were synthesized and their antioxidative activities were evaluated *in vitro* by determination of the levels of malondialdehyde and hydrogen peroxide. Results clearly exhibited that resveratrol and its analogs had various potencies in inhibiting lipid peroxidation in rat brain, kidney, and liver homogenates and rat erythrocyte hemolysis. Several polyhydroxystilbenes were found to be more active than resveratrol in these models, and structure-activity relationship studies on polyhydroxystilbenes are described in this paper.

1. Introduction

Free radicals, particularly reactive oxygen species (ROS), mediated damage has been related to a wide range of pathological disturbance such as brain dysfunction, cancer and cardiovascular disease [1, 2]. For instance, hydroxyl radicals (•OH) induce base modification and DNA strand breaking, which may be a serious event in connection with mutation and carcinogenesis [3–5]. The oxidative damage to membrane polyunsaturated fatty acids (i.e. lipid peroxidation) involves different kinds of radicals such as •OH, lipid radicals, and lipid peroxyl radicals [6]. These radicals are implicated in the etiology of aging and diseases including tumor, multiple sclerosis, Parkinson disease, senile dementia, autoimmune disease and asbestosis [7]. However, free-radical scavengers such as lipid antioxidants could block free radicals from undergoing initiation or propagation of the lipid peroxidation. Therefore, it is very important to search compounds with potent antioxidative activity but low cytotoxicity.

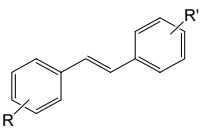
Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene), a naturally occurring product emerged from grapes and various other plants, was found to be antioxidative, antiphlogistic, antitumourous, antimutagenic [8–12], and to be an inducer of phase II drug-metabolizing enzymes [13]. As a phenolic compound, resverstrol contributes the antioxidative potential of red wine and thereby may play a role in the prevention from human cardiovascular diseases. Moreover, as a phytoestrogen, resveratrol may provide cardiovascular protection. Encouragingly, there exists a kind of compounds termed polyhydroxystilbenes, resveratrol analogs, which are widely distributed in the nature and many of them could be used as antioxidative, antifungal, ichthyotoxic, and antileukemic agents [14–16]. We were promoted to explore antioxidative and free radical scavenging activities of resveratrol and its analogs. In the present work, resveratrol and its analogs, polyhydroxystilbenes (henceforth called OH-stilbenes), were synthesized and their antioxidative activities were *in vitro* assessed by determining of the levels of malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in rat brain, kidney and liver homogenates and in rat erythrocyte (RBC) hemolysis. From the results we discuss the efficiency and structure-activity relationship of the antioxidant action of resveratrol and its analogs in these models.

2. Investigations, results and discussion

Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene (**6**) and OH-stilbenes: 4-hydroxy-*trans*-stilbene (**1**), 4,4′-dihydroxy-*trans*-stilbene (**2**), 3,5-dihydroxy-*trans*-stilbene (**3**), 2,4-dihydroxy-*trans*-stilbene (**4**), 3,4-dihydroxy-*trans*-stilbene (**5**), 2,4,4′-trihydroxy-*trans*-stilbene (**7**), as listed in Table 1, were synthesized by literature methods [17–20]. Structural identifications of resveratrol and OH-stilbenes were characterized by NMR and MS.

In the presence of various amounts of resveratrol or other OH-stilbenes lipid peroxidation in rat brain, kidney and liver homogenates was induced by Fe^{2+}-VitC or allowed autooxidation. Levels of MDA were detected on the photospectrometer. All the measurements were run under the same conditions and according to the same procedure. The MDA level, used as an autooxidation index of lipid peroxidation in tissues, was applied herein to screen the effect of resveratrol or its analogs on antioxidative activities towards lipid peroxidation in rat brain, kidney and liver homogenates. The data is shown in Tables 2 and 3. Representative histograms are shown in Fig. 1. Histograms very clearly demonstrated effects of resveratrol and its analogs on the Fe^{2+}-VitC induced lipid peroxidation in rat brain, kidney and liver homogenates. Generally, OH-stilbenes **2**, **5**, **6** (resveratrol) and **7** exhibited remarkable effects on lowering MDA levels in rat brain, kidney and liver homogenates. However, effects of OH-stilbenes **1** and **4** were much less than those of **2**, **5**, **6** (resveratrol) and **7**. Furthermore, OH-stilbene **3** has almost no effect in rat brain homogenates at lower concentrations. For each OH-stilbene its activity featured a dose-dependence. The overall tendency was that the effect was enhanced with increasing amount of OH-stilbenes. Data in Tables 2 and 3 allow to order the inhibitory ability of resveratrol and its analogs in the model as follows: **5** > **7** > **2** > resveratrol

Table 1: Chemical structure of resveratrol and its analogs



OH-Stilbene	R	R′
1	4-OH	H
2	4-OH	4′-OH
3	3,5-$(OH)_2$	H
4	2,4-$(OH)_2$	H
5	3,4-$(OH)_2$	H
6 (Resveratrol)	3,5-$(OH)_2$	4′-OH
7	2,4-$(OH)_2$	4′-OH

Table 2: Effects of resveratrol and its analogs on the Fe^{2+}-VitC induced level of MDA in rat brain, kidney and liver homogenates

Compd.	Conc. (μmol/l)	Brain		Liver		Kidney	
		MDA formation (OD532 nm)	Inhibition (%)	MDA formation (OD532 nm)	Inhibition (%)	MDA formation (OD532 nm)	Inhibition (%)
Control	0	0.482 ± 0.003	–	0.411 ± 0.008*	–	0.325 ± 0.005*	–
1	30	0.230 ± 0.003*	52.28	0.116 ± 0.006*	71.78	0.172 ± 0.003*	47.08
	15	0.273 ± 0.005*	43.36	0.276 ± 0.003*	32.85	0.230 ± 0.003*	29.23
	7.5	0.306 ± 0.003*	36.51	0.335 ± 0.003*	18.49	0.316 ± 0.002*	2.77
2	15	0.045 ± 0.005*	90.66	0.130 ± 0.005*	68.37	0.048 ± 0.003*	85.23
	7.5	0.241 ± 0.003*	50.00	0.219 ± 0.003*	46.72	0.173 ± 0.009*	46.77
	3.75	0.382 ± 0.011*	20.75	0.341 ± 0.007*	17.03	0.296 ± 0.004*	8.92
3	30	0.355 ± 0.002*	26.35	0.243 ± 0.004*	40.88	0.275 ± 0.005*	15.38
	15	0.423 ± 0.004*	12.24	0.309 ± 0.011*	24.82	0.323 ± 0.004	0.62
	7.5	0.480 ± 0.009	0.41	0.367 ± 0.005*	10.71	0.337 ± 0.003	−3.69
4	30	0.023 ± 0.003*	95.23	0.126 ± 0.003*	69.34	0.071 ± 0.003*	78.15
	15	0.216 ± 0.012*	55.19	0.236 ± 0.007*	42.58	0.198 ± 0.012*	39.08
	7.5	0.315 ± 0.007*	34.65	0.271 ± 0.021*	34.06	0.276 ± 0.005*	15.08
5	15	0.016 ± 0.002*	96.68	0.003 ± 0.002*	99.27	0.035 ± 0.007*	89.23
	7.5	0.025 ± 0.003*	94.81	0.130 ± 0.007*	68.37	0.064 ± 0.005*	79.38
	3.75	0.041 ± 0.005*	91.49	0.301 ± 0.008*	26.76	0.176 ± 0.004*	45.85
6 (Resveratrol)	30	0.029 ± 0.004*	93.98	0.080 ± 0.003*	80.54	0.057 ± 0.003*	82.46
	15	0.081 ± 0.008*	83.20	0.204 ± 0.005*	50.36	0.123 ± 0.002*	62.15
	7.5	0.288 ± 0.005*	40.25	0.306 ± 0.016*	25.55	0.216 ± 0.002*	33.54
7	15	0.027 ± 0.004*	94.40	0.031 ± 0.004*	92.46	0.040 ± 0.003*	87.69
	7.5	0.151 ± 0.003*	68.67	0.176 ± 0.010*	57.18	0.112 ± 0.002*	65.54
	3.75	0.277 ± 0.003*	42.53	0.304 ± 0.006*	26.03	0.235 ± 0.000*	27.69

All compounds were dissolved in DMSO. The values are mean ±SD (n = 3). *: $P < 0.05$ or less compared with the control by Students' t test

(**6**) > **4** > **1** > **3**. The individual inhibitory efficiency shown in Tables 2 and 3 for each OH-stilbene was different from one to another tissues homogenates. For instance, effects of resveratrol in brain, kidney and liver homogenates (Fig. 2) shows an order as follows: brain > kidney > liver. However, the order for OH-stilbene **5** was: liver > brain > kidney; for OH-stilbene **2**, brain > kidney > liver.

Table 3: Effects of resveratrol and its analogs on the autooxidation MDA level in rat brain, kidney, and liver homogenates

Compd.	Conc. (μmol/l)	Brain		Liver		Kidney	
		MDA formation (OD532 nm)	Inhibition (%)	MDA formation (OD532 nm)	Inhibition (%)	MDA formation (OD532 nm)	Inhibition (%)
Control	0	0.286 ± 0.007	–	0.130 ± 0.004	–	0.161 ± 0.003	–
1	30	0.065 ± 0.005*	77.27	0.071 ± 0.003*	45.38	0.063 ± 0.005*	60.87
	15	0.234 ± 0.005*	18.18	0.122 ± 0.023	6.15	0.106 ± 0.003*	34.16
	7.5	0.267 ± 0.004*	6.64	0.133 ± 0.007	−2.31	0.129 ± 0.001*	19.88
2	7.5	0.042 ± 0.003*	85.31	0.014 ± 0.003*	89.23	0.061 ± 0.003*	62.11
	3.75	0.128 ± 0.005*	55.24	0.055 ± 0.005*	57.69	0.096 ± 0.002*	40.37
	1.87	0.215 ± 0.009*	24.83	0.121 ± 0.003*	6.92	0.134 ± 0.002*	16.77
3	30	0.172 ± 0.003*	39.86	0.107 ± 0.003*	17.69	0.075 ± 0.003*	53.42
	15	0.280 ± 0.008*	2.10	0.126 ± 0.005*	3.19	0.119 ± 0.002*	26.09
	7.5	0.301 ± 0.003	−5.24	0.137 ± 0.004	−5.38	0.137 ± 0.004*	14.91
4	15	0.070 ± 0.002*	75.52	0.014 ± 0.003*	89.23	0.069 ± 0.006*	57.14
	7.5	0.200 ± 0.003*	30.07	0.075 ± 0.005*	42.31	0.101 ± 0.003*	32.27
	3.75	0.229 ± 0.011*	19.93	0.107 ± 0.015*	17.69	0.130 ± 0.005*	19.25
5	3.75	0.008 ± 0.002*	97.20	0.008 ± 0.004*	94.19	0.059 ± 0.002*	63.35
	1.87	0.017 ± 0.003*	94.06	0.079 ± 0.003*	39.23	0.083 ± 0.002*	48.45
	0.94	0.030 ± 0.002*	89.51	0.107 ± 0.007*	17.69	0.111 ± 0.009*	31.06
6 (Resveratrol)	15	0.033 ± 0.004*	88.46	0.008 ± 0.002*	93.85	0.068 ± 0.005*	57.76
	7.5	0.086 ± 0.007*	69.93	0.042 ± 0.003*	67.69	0.096 ± 0.003*	40.37
	3.75	0.177 ± 0.003*	38.11	0.096 ± 0.004*	26.15	0.110 ± 0.003*	31.68
7	7.5	0.026 ± 0.003*	90.91	0.008 ± 0.002*	93.85	0.054 ± 0.012*	66.46
	3.75	0.037 ± 0.005*	87.06	0.054 ± 0.004*	58.46	0.086 ± 0.005*	46.58
	1.87	0.205 ± 0.015*	28.32	0.109 ± 0.005*	16.15	0.114 ± 0.003*	29.19

All compounds were dissolved in DMSO. The values are mean ±SD (n = 3). *: $P < 0.05$ or less compared with the control by Students' t test

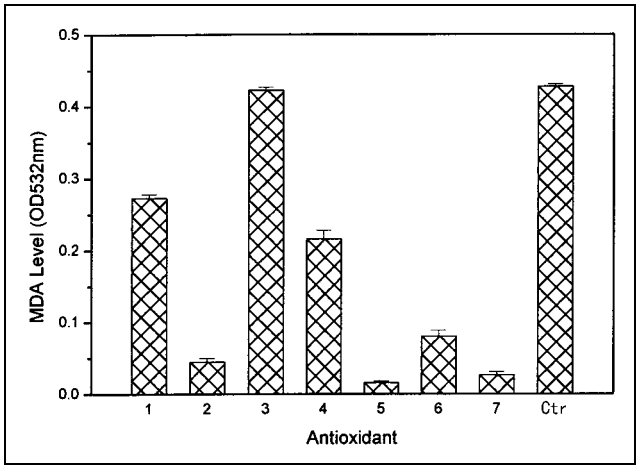


Fig. 1: Effect of resveratrol and its analogs (15 μmol · l^{-1}) on the Fe^{2+}-VitC induced MDA level in rat brain homogenates

In the presence of various amounts of resveratrol or its analogs the H_2O_2-caused RBC hemolysis was carried out and the level of H_2O_2 in RBC probes was detected on the photospectrometer at 415 nm. Data is shown in Table 4. Representative histograms were drawn in Fig. 3. The H_2O_2-caused RBC hemolysis mainly reflects the extent of lipoperoxidation of RBC membrane [21]. The level of H_2O_2 can be applied to evaluate the effect of antioxidants on the RBC hemolysis. Results clearly revealed that resveratrol and its analogs could antagonize hemolysis induced by H_2O_2 in a dose-dependent manner. With increasing the amount of OH-stilbenes the H_2O_2-level was efficiently lowered. Compared to results in rat brain, kidney and liver homogenates, the inhibitory ability order of resveratrol and its analogs in RBC hemolysis was: **5** > **7** > **2** > resveratrol (**6**) > **4** > **3** > **1**. The sole difference was **3** > **1** in the present case instead of **1** > **3** in the above cases.

Ranking the antioxidative activity of resveratrol and its analogs in one assay, in general trend, follows the ranking in the other assay but with minor differences. For instance, OH-stilbene **3** is more active than OH-stilbene **1** in antagonizing hemolysis induced by H_2O_2 while it is less active than OH-stilbene **1** in the assay of the autooxidation and Fe^{2+}-VitC induced lipid peroxidation in rat brain, kidney and liver homogenates. This is probably attributed

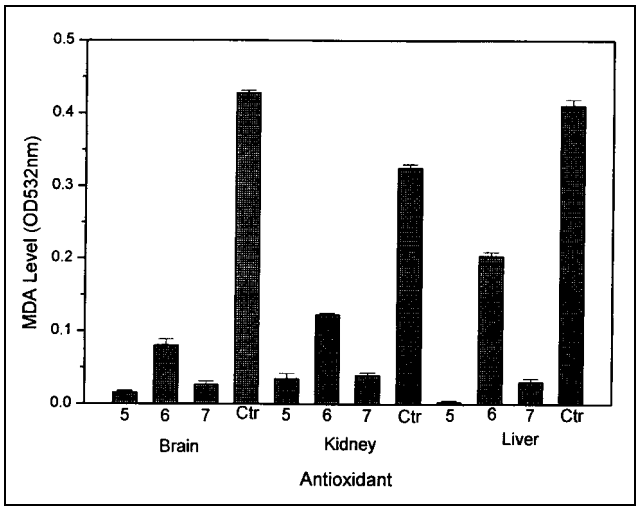


Fig. 2: Effects of OH-stilbenes **5**, **6**, **7** (15 μmol · l^{-1}) on the Fe^{2+}-VitC induced MDA level in rat brain, kidney, and liver homogenates

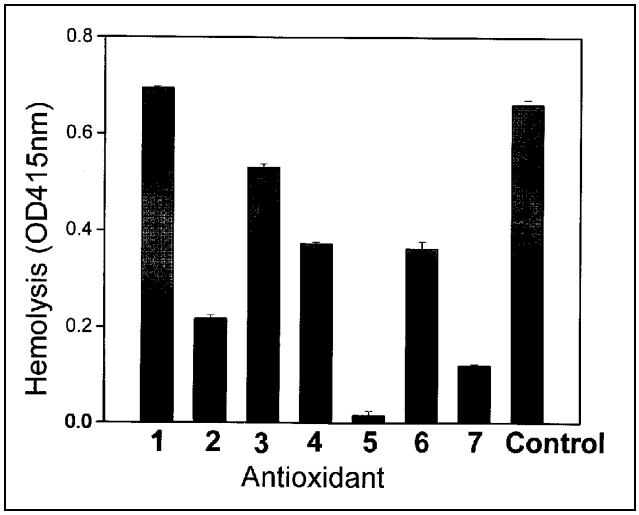


Fig. 3: Effects of resveratrol and its analogs (4 μmol · l^{-1}) on the RBC hemolysis induced by H_2O_2

to a different mechanism involved in each assay process. It is interesting to note that the inhibitory activity of resveratrol and its analogs in the lipid peroxidation assay using rat brain homogenates was similar to that in a similar assay using rat kidney and liver homogenates. It seems to be referred to the same mechanism involved in the two assays.

Experiments demonstrated that all the OH-stilbenes were able to remarkably lower MDA levels and to antagonize RBC hemolysis, which indicated that they possess antioxidative activity. Among them, the most potent one is OH-stilbene **5**. The reason for the observation would be:

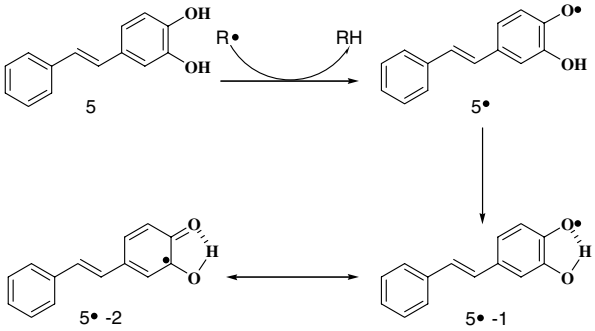
Firstly, that aryloxy radicals of **5**, **5**•, derived via H-abstraction by oxygen radical species such as •OH could be

Table 4: Effects of resveratrol and its analogs on RBC hemolysis induced by H_2O_2

Compd.	Concentration (μmol/l)	Hemolysis extent (OD415 nm)	Inhibition (%)
Control	0	0.661 ± 0.010	–
1	12	0.245 ± 0.004*	62.93
	8	0.353 ± 0.005*	46.60
	4	0.695 ± 0.003*	−5.14
2	6	0.120 ± 0.005*	81.85
	4	0.217 ± 0.007*	67.17
	2	0.603 ± 0.004*	8.77
3	6	0.326 ± 0.003*	50.68
	4	0.529 ± 0.008*	19.97
	2	0.663 ± 0.005*	−0.30
4	6	0.143 ± 0.005*	78.37
	4	0.372 ± 0.003*	43.72
	2	0.640 ± 0.004*	3.18
5	4	0.016 ± 0.009*	97.58
	2	0.069 ± 0.003*	89.56
	1	0.198 ± 0.007*	70.04
6 (Resveratrol)	6	0.024 ± 0.004*	86.37
	4	0.362 ± 0.015*	45.23
	2	0.625 ± 0.006*	5.45
7	6	0.049 ± 0.005*	92.59
	4	0.120 ± 0.003*	81.85
	2	0.596 ± 0.004*	9.83

All compounds were dissolved in DMSO. The values are mean ±SD (n = 3).
*: $P < 0.05$ or less compared with the control by Students' t test

Scheme



Stabilizing mechanisms of free radicals derived from the OH-stilbene **5**

stabilized by forming an intramolecular hydrogen bond with neighboring OH group (**5**• -1 in the Scheme). Secondly, **5**• -1 could form an o-benzoquinonoid (**5**• -2 in Scheme) structure through resonance [22], in which the odd electron delocalizes itself over multiple C−C double bonds and thus leads to stabilize much the radical.

Concerning the relationship between structures and antioxidative activities, results indicate that antioxidative activities of resveratrol and its analogs are affected by several factors: a) the formation of an intramolecular hydrogen bond, which is favorable for stabilizing H-abstracted radicals through resonance; b) the location of OH groups on benzene rings. OH-stilbenes are not active as antioxidants unless the OH-substituent at either the ortho or para position, which brings about the enhancement of the electron density on hydroxyl groups and the reduction of the oxygen-hydrogen band energy [19, 23]. As a result, it gives rise to increase the reactivity towards free radicals; c) the number of OH groups on benzene rings. The more the number of OH groups a OH-stilbene molecule possesses, the higher the •OH scavenging reactivity the OH-stilbene exhibits.

The data on in vitro biological evaluation of resveratrol and its analogs exhibited the higher potency of OH-stilbenes in inhibiting lipid peroxidation in rat brain, kidney and liver homogenates and RBC hemolysis. OH-stilbenes **2**, **4**, **5** and **7** were found to have a superior or comparable activity to resveratrol. It could be concluded that OH-stilbenes possess remarkable antioxidative activities and they could be referred to as a kind of potential antioxidant drugs, worth to be further studied.

3. Experimental

3.1. Reagents

2-Thiobarbituric acid (TBA), methoxy-substituted benzyl alcohols and L-ascorbic acid were obtained from Sigma. All other chemicals were of the highest quality available.

3.2. Animals

Male Wistar rats (150–200 g) were purchased from The Animal Breeding Center, Lanzhou Medical College. The rats were housed under normal laboratory conditions (at 21 ± 2 °C, with a $12_{A.M.}-12_{P.M.}$ light-dark cycle) with free access to standard rodent chow and water.

3.3. Syntheses

3.3.1. Synthesis of resveratrol

4-Methoxybenzyl alcohol (5 g) was dissolved in dry benzene, and excess dry HBr gas was bubbled into the solution with stirring over a period of 20 min. The solution was then heated to 78 °C and allowed to stand for 1 h. Water (50 ml) was added to remove HBr and the benzene layer was dried by $MgSO_4$. Evaporation of benzene gave a quantitative yield of slightly colored oil of 4-methoxybenzyl bromide. 4-Methoxybenzyl bromide (3.8 g) was heated with excess triethyl phosphate (4.5 ml) to 130 °C until the evolution of ethyl bromide ceased. Then, the solution was cooled to 0 °C and 25 ml of N,N-dimethylformamide and 1.1 g of sodium methoxide were added to it. Afterwards, 2.1 g of 3,5-dimethoxybenzaldehyde was added. The solution was allowed to stand at room temperature for 1 h. The reaction mixture was then heated to 100 °C and allowed to stand at this temperature for 1 h, and kept at room temperature overnight. Water-methanol (2 : 1, v/v, 40 ml) was added. The precipitated product, 3,4′,5,-trimethoxy-*trans*-stilbene, was collected by filtration and washed with water. The purity of this stilbene was detected by TLC (hexane : ethyl acetate = 4 : 1, v/v). The yield was 2.9 g (87%). Excess pyridine hydrochloride (11 g) and 3,4′,5,-trimethoxy-*trans*-stilbene (2 g) were mixed and heated to 190 °C for 4 h. The hot dark syrup was poured into 50 ml of 2 N HCl. The reaction mixture was extracted with ethyl acetate (3×100 ml). The ethyl acetate layer was dried with $MgSO_4$ and ethyl acetate was then removed under reduced pressure. The residual was purified by CC on silica gel eluted with chloroform-methanol (15 : 1, v/v) to get resveratrol 0.71 g (42%).

3.3.2. Synthesis of OH-stilbenes

OH-stilbenes were synthesized in the same manner as resveratrol using the corresponding benzyl alcohol and benzaldehyde instead of 4-methoxybenzyl alcohol and 3,5-dimethoxybenzaldehyde.

3.4. Assay of lipid peroxidation using rat brain, kidney and liver homogenates

The rat brain, or kidney, or liver, from rats was weighed and homogenized as a 5% (w/v) tissue homogenate in ice-cold Tris-KCl buffer (20 mM, pH 7.4). An appreciate amount of resveratrol or OH-stilbenes was added to 1.0 ml of the homogenate and the sample (suspension) rehomogenized, followed incubation at 37 °C for 10 min. 100 Microliter of Fe^{2+}-VitC solution ($FeSO_4$/ascorbic acid = 50/50 μmol l^{-1}) was added. After 0.5 h incubation, the reaction was stopped by addition of 1.0 ml trichloroacetic acid (TCA, 28%, w/v) and 1.5 ml TBA (0.67%, w/v) in succession. The solution was then warmed for 15 min in a boiling water bath. After cooling, the optical density (OD) value of the solution, which was applied to estimate the level of MDA, was measured at 532 nm on a Hitachi 557 spectrophotometer [24, 25]. The spontaneous (i.e. autooxidation) MDA generations in rat brain, kidney, and liver homogenates were detected after 2 h incubation with resveratrol or OH-stilbenes but without Fe^{2+}-VitC. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = (\text{A-A}_1)/\text{A} \times 100\%$$

where A was the OD in the control, and A_1 was the OD of the probe.

3.5. Assay of RBC hemolysis

Rat blood was obtained from rats by cardiac puncture and collected in heparinized tubes. RBCs were separated from plasma and the buffy coat. The probe of RBC was washed three times with 10 volumes of 0.15 M NaCl and made into a 0.5% (w/v) suspension. One Milliliter of RBC suspension was incubated with H_2O_2 (100 mmol l^{-1}) at 37 °C for 1 h after adding resveratrol or OH-stilbenes. The probe was diluted with 5 volumes of normal saline and the OD of the supernatant was measured at 415 nm under 0.5 cm optical path, which was applied to estimated to the level of H_2O_2 in the probe. The OD value in the control was defined as 100%. The hemolysis extent was calculated by referring to the control [26, 21]. The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio (\%)} = (\text{A-A}_1)\ /\text{A} \times 100\%$$

where A was the OD in the control, and A_1 was the OD of the probe.

Acknowledgement: The authors thank The Natural Scientific Foundation of China for the financial support (The grant No. 20072013)

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Received December 12, 2001
Accepted January 12, 2002

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