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Antioxidant properties of phenolic diterpenes from *Rosmarinus officinalis*[1]

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KEY WORDS antioxidants; LDL lipoproteins; free radicals; carnosol

ABSTRACT

AIM: To investigate the inhibition capacities of carnosol, rosmanol, and epirosmanol, which are phenolic diterpenes from *Rosmarinus officinalis*, to oxidized low-density lipoprotein (LDL) formation in human blood and detect their scavenging activities to lipid free radical and superoxide anion *in vitro*. **METHODS**: The antioxidant activities which were expressed with the inhibilities to lipid free radicals in the membrane lipid of cell and oxidized LDL formation were evaluated by TBARS assay and ESR method. The inhibition on the Cu^{2+}-mediated oxidization of *apo* B formation in LDL was investigated by fluorescence spectroscopy. **RESULTS**: Carnosol, rosmanol, and epirosmanol had an inhibitory activity to lipid peroxidation and oxidized *apo* B formation in human bloods LDL. The IC_{50} were 7 - 10 μmol/L. The antioxidant mechanism was related to the scavenging activities to lipid free radical. **CONCLUSION**: carnosol, rosmanol, and epirosmanol showed the activity in inhibiting LDL oxidation.

INTRODUCTION

More and more evidence suggests that the oxidation of low density lipoprotein (LDL) may play a pivotal role in the pathogenesis of atherosclerosis[1]. The exact mechanism by which LDL undergoes oxidation *in vivo* is not yet fully understood, but increasing experimental data suggest that it involve free radical formation during LDL oxidation[2].

Rosmary (*Rosmarinus officinalis* L), an evergreen shrub, is one of the herb spices of the family *Labiatae*. It was cultivated in Mediterranean first, then transplanted to China in Jin Dynasty, but cultivated in all of the world now. At present, there is a large source of rosemary in Yunnan Province of China. Diterpenoids, flavonoids, triterpenoids, essentials and phenolic acids are their main constituents. Its extracts have been developed and used in medicine, cosmetics, and keeping foods fresh, especially the phenolic diterpenoids fraction in total extracts shows prominent bio-activities of antioxidant, antitumor, and anti-HIV[3,4]. Carnosol, rosmanol, and epirosmanol from *Rosmarinus officinalis* have phenolic diterpenes structure. It was reported that they could inhibit mitochondria and microsomal lipid peroxidation induced by NADH or NADPH[5]. But, the molecular mechanism of them as a protector against LDL oxidation and vascular diseases is unclear. The present work was conducted to evaluate the activity of carnosol, rosmanol, and epirosmanol in inhibiting LDL oxidation.

MATERIALS AND METHODS

Materials Carnosol, rosmanol and epirosmanol (Fig 1) were obtained from the Department of Natural Medicines in School of Pharmaceutical Sciences, Peking University, China. The extracted process was described in literature[6], 98 % purification (HPLC detection). The compounds were fully dissolved in Me_2SO, then diluted with phosphate buffer solution (PBS) and finally there was 3 % Me_2SO in the compound solution. Spin trap 4-POBN, nitrobluetetrazolium (NBT), xanthine (X) and xanthine oxidase (XOD) were purchased from Sigma company in USA. Other agents were of AR grade. Human LDL were isolated from plasma of normal volunteers in the density range of 1.019 - 1.063

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kg/L and oxidized by incubating with $CuSO_4$ 10 μmol/L at 25 ℃ for 24 h[7]. The oxidation reaction was stopped by adding 100 μmol/L ethylenedinitrolotetraacetic acid (EDTA). The microsomes were prepared from mouse liver[8]. The membrane was from the fresh blood[9].

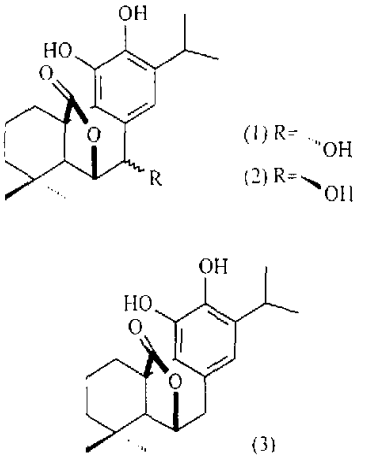

Fig 1. Structure of rosmanol (1), epirosmanol (2), and carnosol (3).

Thiobarbituric acid reactive substance (TBARS) assay[10] LDL (1.2 mL, 0.64 g/L) that was oxidized by $CuSO_4$ (10 μmol/L) was mixed with various concentration of phenolic diterpenes and diluted with 1mL of 20 % trichloroacetic acid. After adding the thiobarbituric acid (1 mL of 0.8 %), the mixture was heated at 100 ℃ for 20 min. The peak absorbance at 532 nm of the solution which was read. The IC_{50} (95 % confidence limits) were calculated by probit analysis (SAS 6.12).

Fluorescence detection The impact on LDL-oxidized can be assessed by evaluating the inhibition of carnosol, rosmanol, and epirosmanol on the oxidized *apo* B formation in LDL. When LDL unsaturated fatty acid undergoes oxidation, reactive aldehyde is formed that bind to *apo* B. Any inhibition to the formation of reactive aldehyde will blocked consequently the modification of *apo* B. We recorded the fluorescence spectrum of LDL subjected to oxidative conditions in the absence or presence of carnosol, rosmanol and epirosmanol. LDL (0.64 g/L) with $CuSO_4$ (10 μmol/L) and various concentration of phenolic diterpenes were diluted with PBS (pH 7.4) to give a final concentration of LDL 120 mg/L. The extent of aldehyde-modified lysine in oxidized LDL was monitored by determining the fluorescence of native or modified LDL samples, using an excitation wavelength of 360 nm and monitoring emission fluorescence at 420 nm with a DR-3 fluorescence spectrophotometer.

Superoxide anion and lipid free radicals scavenging activity Superoxide anion was generated by X/XOD and measured by the NBT reduction method[11]. The absorbance of mixture at 560 nm was recorded after mixt for 10 min. Percentage of scavenging was calculated as following: scavenging capacity = $(A_0 - A_s)/A_0 \times 100$ %. Here, A_0, A_s are the absorbances with or without addition of compounds, respectively. The inhibiting activity of compounds to lipid free radical (L·) was investigated, comparing with a standard generated system in which PBS as a control. L· was generated in the system of mouse liver microsomes after adding 6 % H_2O_2 and measured by spin traping-ESR method. The ESR signal of lipid free radicals traped by 4-POBN (POBN-L·) was recorded by ESR spectrometer[12]. The changes of signal strengths of POBN-L· in the system that was interacted respectively with carnosol, rosmanol, and epirosmanol were detected as evaluating the inhibiting activity to L·. ESR spectra were measured within 5 min after reagents mixing at room temperature using a Bruker ESP-300 spectrometer (Germany) operating at 9.74 GHz with 100 kHz field modulation. A scan range of 20 mT, a modulation amplitude of 0.20 mT, a microwave power of 10 mW, a receiver gain of 5×10^5, time constants of 160 ms, and a scan time of 200 s were used for all spectra.

RESULTS

Inhibition on lipid peroxidation in the cell membrane and human LDL Carnosol, rosmanol, and epirosmanol inhibited TBARS formation in Cu^{2+}-mediated LDL oxidation. The antioxidant IC_{50} value was evaluated as the absorbance of the antioxidant concentration that corresponding to 50 % decrease of the TBARS of LDL-Cu^{2+} at 532 nm and listed in Tab 1.

Inhibition of the formation of *apo* B in LDL Oxidized *apo* B formation in LDL can be detected by the fluorescence properties which attributed from the formation of Schiff base products, with excitation and emission maximum at 350 nm and 420 nm respectively[13]. This represented adduct formation between reactive aldehydes and lysine residues of *apo* B. Carnosol, rosmanol, and epirosmanol inhibited this

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Tab 1. Inhibition on Cu^{2+}-induced LDL oxidation and lipid free radicals in mouse liver microsomes by carnosol, rosmanol, epirosmanol, and α-tocopherol. Experimental groups in ESR: liver microsome + 6 % H_2O_2 + compound (100 nmol/L) + 4-POBN (200 mmol/L); $n = 3$ independent experiments. 95 % confidence limits are in parentheses.

Compound	IC_{50}/μmol·L^{-1} in LDL	IC_{50}/μmol·L^{-1} in cell membrane	h[1] (au)	Inhibition to L·/%
Carnosol	10.015 (8.726 - 12.041)	4.530 (2.849 - 7.465)	2.15	34.84
Rosmanol	7.041 (5.648 - 9.264)	2.505 (1.112 - 4.728)	1.95	40.90
Epirosmanol	8.227 (6.546 - 11.162)	3.511 (2.130 - 6.146)	2.45	27.27
α-Tocopherol	48.011 (3.000 - 93.000)		2.75	16.66
Control[2]			3.3	0

1) The signal height of 4-POBN/L, au: arbitrary unit; 2) Control: liver microsome + 6 % H_2O_2 + 4-POBN (200 mmol/L); others: liver microsome + 6 % H_2O_2 + compound (2×10^{-5} mmol/L) + 4-POBN (200 mmol/L).

apo B modification that is related to the concentration of compounds. Carnosol, rosmanol, and epirosmanol have an inhibition tendency at low concentration (< 20 μmol/L), only rosmanol can keep the inhibition above 20 μmol/L. Another two showed a little bit increasing of fluorescence absorbance and then keeped constant above 20 μmol/L. (Fig 2). These indicated that they could show a main effected inhibition at low concentration of compounds. It is not found that they quench the endogenous fluorescence in LDL under the researching condition.

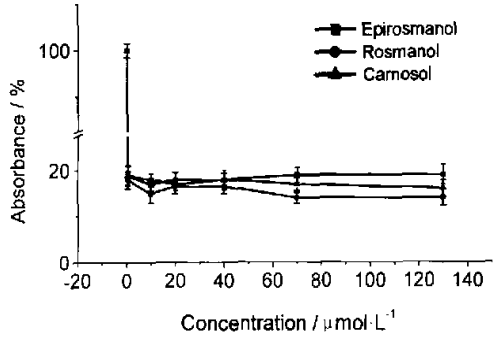

Fig 2. Fluorescence of LDL (12 mg/L) incubated with Cu^{2+} 5 μmol/L in the presence of the phenolic diterpenes. $n = 3$. $\bar{x} \pm s$. ▲: LDL plus Cu^{2+} plus carnosol of different concentration; ●: LDL plus Cu^{2+} plus rosmanol of different concentration; ■: LDL plus Cu^{2+} plus epirosmanol of different concentration.

Effect on lipid free radical and NBT reduction by superoxide anion generated from X/XOD

The ESR signal of 4-POBN was shown in Fig 3 and the parameters were similar to that in reference[12]. The inhibition activities of carnosol, rosmanol, and epirosmanol to L· were listed in Tab 1. Carnosol, rosmanol, and epirosmanol did not inhibit the activity of XOD, but scavenged superoxide anion at low concentration. Rosmanol showed an increasing of scavenging action when its concentration was increased, but another two had a little decreasing of scavenging action when the concentration is above 0.2 μmol/L (Fig 4).

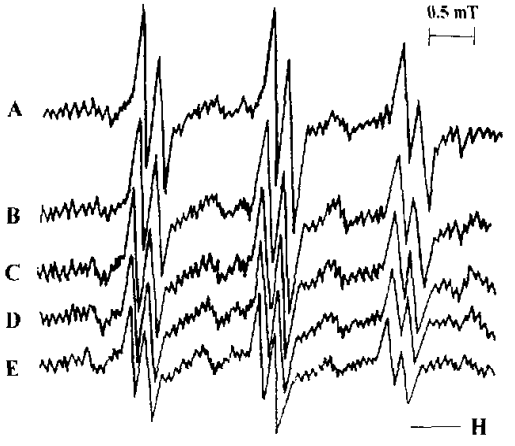

Fig 3. ESR Spectrum of POBN-L· adduct. The inhibition potency was calculated by the peak-height ratio in the low field with or without any antioxidant in the research systems. A: control: liver microsome + 6 % H_2O_2 + 4-POBN (200 mmol/L); B: liver microsome + 6 % H_2O_2 + α-tocopherol (20 nmol/L) + 4-POBN (200 mmol/L); C: liver microsome + 6 % H_2O_2 + epirosmanol (20 nmol/L) + 4-POBN (200 mmol/L); D: liver microsome + 6 % H_2O_2 + rosmanol (20 nmol/L) + 4-POBN (200 mmol/L); E: liver microsome + 6 % H_2O_2 + carnosol (20 nmol/L) + 4-POBN (200 mmol/L).

DISCUSSION

The experimental results using several techniques

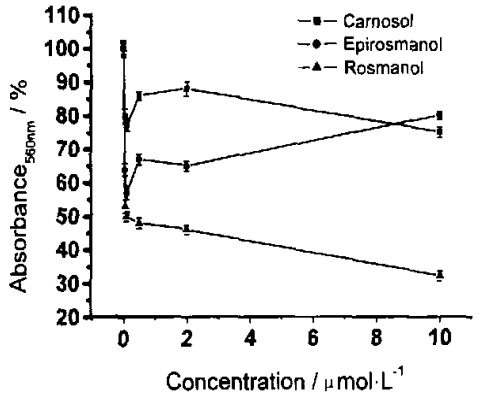

Fig 4. Effects on the reduction of NBT by superoxide anion generated from the X/XOD system.

suggest that carnosol, rosmanol and epirosmanol showed capacities of inhibiting LDL or cell membrane oxidation mediated by copper. The results indicated that they could inhibit the formation of aldehydes peroxidation products in LDL and cell membrane. Fluorescence research also showed that they inhibited the interaction of lipid oxidation products and apolipoprotein at low concentration. From Tab 1, they all had strong inhibition not only to the formation of lipid oxidation products but also to the process of oxidative modification of *apo* B in LDL.

The mechanism by which carnosol, rosmanol, and epirosmanol inhibit LDL oxidation is probably a synergistic effect of substance such as blocking the direct modification to *apo* B and inhibiting an adduct formation between reactive aldehydes and lysine residues of *apo* B. In addition to that, the important mechanisms of antioxidant activity are strongly due to the direct scavenging to lipid free radical and superoxide anion. Under this oxygen-derived radical generation system, the yield of lipid free radical and superoxide anion was actually more considerable than the others in clinical pathological condition[14]. Obviously, carnosol, rosmanol and epirosmanol are very sensitives for preventing lipid free radical and superoxide-induced damages within a micromolar concentration region.

At present, There is functional product of the extracts from Rosemary as preventing cardiovascular diseases especially arteriosclerosis in America market. It is considered that, probably, the action is closed to the antioxidant properties of phenolic diterphenoid. Our results suggest that the carnosol, rosmanol and epirosmanol of rosemary extracts play a very important action in preventing cardiovascular diseases.

From IC_{50} data in Tab 1, there are not significant difference among the carnosol, rosmanol, and epirosmanol, but there is a difference in scavenging lipid free radical. This is probably related to the their structure. All of them include a ortho-position diphenolic groups that is easy to give out a H· to form a ortho-position diquinion. The H· will react with other free radicals either from endogenous or exogenous to form stable compound. The number of OH group is probably related to the activity of the antioxidant results in which rosmanol is stronger than carnosol.

REFERENCES

1 Hulthe J, Bokemark L, Wikstrand J, Fagerberg B. The *metabolic syndrome, LDL particle size, and atherosclerosis*: the atherosclerosis and insulin resistance (AIR) study. Arterioscler Thromb Vase Biol 2000; 20: 2140-7.

2 Thomas R, Stocker R. Molecular action of vitamin E in lipoprotein oxidation: implications for atherosclerosis. Free Radic Biol Med 2000; 28: 1795-805.

3 Aruoma OI, Spencer JP, Rossi R, Aeschbach R, Khan A, Mahmood N, *et al*. An evaluation of the antioxidant and antiviral action of extracts of rosemary and Provencal herbs. Food Chem Toxicol 1996; 34: 449-56.

4 Plouzek CA, Ciolino HP, Clarke R, Yeh GC. Inhibition of P-glycoprotein activity and reversal of multidrug resistance *in vitro* by rosemary extract. Eur J Cancer 1999; 35: 1541-5.

5 *Haraguchi H, Saito T, Okamura N, Yagi A. Inhibition of* lipid peroxidation and superoxide generation by diterpenoids from Rosmarinus officinalis. Planta Med 1995; 61: 333-6.

6 Nakatni N, Inatani R. Two antioxidative diterpenes from rosemary (*Rosmarinus Officinus* L) and a revised structure for rosmanol. Agric Biol Chem 1984; 48: 2081-5.

7 Sparrow CP, Parthasarathy S, Steinberg D. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. J Biol Chem 1989, 264: 2599-604.

8 Guido RMMH, Aalt B. Protection against lipid peroxidation by a microsomal glutathione-dependent labile factor. FEBS 1983; 159: 24.

9 *Waugh RE, Mantalaris A, Bauserman RG, Hwang WC, Wu* JH. Membrane instability in late-stage erythropoiesis. Blood 2001; 97: 1869-75.

10 Esterbauer H, Gebicki J, Puhi H, Jurgens G. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. Free Radic Biol Med 1992; 13: 341-90.

11 Ogawa K, Kanematsu S, Asada K. Generation of superoxide anion and localization of CuZn-superoxide dismutase in the vascular tissue of spinach hypocotyls: their association with lignification. Plant Cell Physiol. 1997; 38: 1118-26.

12 Lu JF, Wang K, Gua YS, Zhang YT, Xu P, Liu HY.

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E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Study on the free radical mechanism of hepatotoxicity by pt(II) complex. Prog Nat Sci 1997; 7: 695 – 700.
13 Steinbrecher UP, Witztum JL, Parthasarathy S, Steinberg D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. Correlation with changes in receptor-mediated catabolism. Arteriosclerosis 1987; 7: 135 – 43.
14 Fridovich I. Oxygen toxicity: a radical explanation. J Exp Biol 1998; 201: 1203 – 9.

迷迭香中二萜酚化合物的抗氧化作用[1]

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关键词 抗氧化剂; 低密度脂蛋白类; 自由基; 鼠尾草酚

目的: 研究迷迭香中二萜酚化合物鼠尾草酚、迷迭香酚和表迷迭香酚对人低密度脂蛋白氧化的抑制作用及其对自由基和超氧阴离子的清除作用. **方法**: 以细胞膜和 LDL 为靶, 用 TBARS 方法和 ESR 方法研究了抗氧化活性. 用荧光方法研究对铜诱导的 LDL 中 *apo* B 蛋白氧化的抑制. **结果**: 鼠尾草酚、迷迭香酚和表迷迭香酚对人血 LDL 中的脂质过氧化和 *apo* B 蛋白的氧化均有抑制作用, IC_{50}值在 7 – 10 μmol/L. 抗氧化机理与其对脂自由基清除活性有关. **结论**: 鼠尾草酚、迷迭香酚和表迷迭香酚能抑制 LDL 氧化.

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