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Original Article

# Protective Effect of *Rosmarinus officinalis* L. Essential Oil against Free Radical-Induced Erythrocyte Lysis

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**Abstract**

The oxidative hemolysis of rat erythrocytes induced by 2,2'-azobis–(2-amidinopropane) (AAPH) and its inhibition by rosemary essential oil was studied. Different concentrations (0.178, 0.357, 0.534 and 0.712 µl/ml) of the essential oil showed no significant hemolysis compared to phosphate buffer solution. AAPH (25 mM and 50 mM) induced hemolysis in a time-dependent manner. Different concentrations of the essential oil inhibited hemolysis induced by 25 mM AAPH. However, in the presence of 50 mM of AAPH, only the two higher concentrations (0.534 and 0.712 µl/ml) of the essential oil inhibited hemolysis. Addition of essential oil 2 or 3 h after incubation with AAPH had no significant effects on the time course of cell lysis. It is concluded that, in addition to its well-established antioxidant effects, rosemary essential oil displays cytoprotective properties.

*Keywords:* AAPH; Hemolysis; *Rosmarinus officinalis*.
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## 1. Introduction

Increasing evidence suggests that free radical-induced oxidative damages lead to various pathological events including coronary heart disease, cancer and aging [1]. In particular, lipid peroxidation in biological membranes has attracted much attention in relation to the deterioration of membrane structure and impairment of enzymatic functions [2].

The oxidation of erythrocyte membranes serves as a model for the oxidative damage of biomembranes [3, 4]. The oxidation of erythrocyte and its ghost's membranes induced by free radicals have also been studied, and it has been found that free radicals generated in the aqueous phase attack the membrane to induce the chain oxidations of lipids and proteins and eventually cause hemolysis [5, 6]. Much interest exists in the possibility that antioxidants reduce the risk of degenerative diseases by inhibiting free radical induced oxidative damage [1]. Therefore, several studies have examined both natural and synthetic antioxidants for the inhibition of lipid peroxidation in membrane systems.

*Rosemarinus officinalis* L. (Labiatae) is a common household plant grown in many parts

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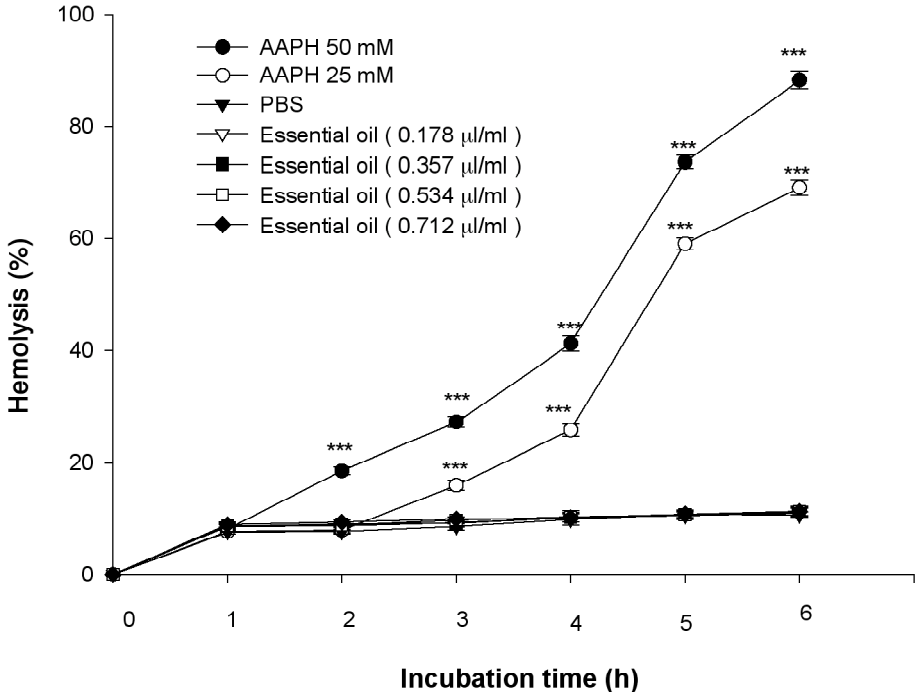


**Figure 1.** Effect of different concentrations of rosemary essential oil and AAPH on erythrocytes (n= 5, mean ± SEM, *** $p<0.001$ compared to PBS).

of the world [7]. Its extracts have been widely used as preservative in food industry due to the antioxidant activity of some of its constituents such as carnosol and carnosoic acid [8]. These compounds have been shown to inhibit the non enzymatic-induced lipid peroxidation in liver microsomes when incubated in the presence of $FeCl_3$ [9]. In isolated rat hepatocytes, an aqueous extract of *R. officinalis* has been shown to reduce *tert*-butylhydroperoxide effects on lipid peroxidation [10].

Based on these precedents, it can be inferred that blocking free radical propagation and lipid peroxidation would protect erythrocytes against the deleterious effects of oxidative stress. In the present study, erythrocytes were oxidized by a water-soluble radical generator 2,2' azobis (2-amidinopropane) hydrochloride (AAPH) and the protective effect of rosemary essential oil on hemolysis was investigated.

## 2. Materials and methods

### *2.1. Plant material and essential oil isolation*

The aerial parts of *R. officinalis* L. were collected in Mashhad, Iran, dried in shade, and then grounded. The plant was identified by the Department of Botany, Ferdowsi University, Mashhad, Iran. A voucher specimen was deposited in the herbarium of the Faculty of Pharmacy, Mashhad (153-1815-06).

The oil was obtained by hydrodistillation using a clevenger-type apparatus for 3 h to give pale yellow oil. It was dried over anhydrous sodium sulfate and stored at 4-8 ºC in the dark. The yield of the extract was 1.5% (v/w).

### *2.2. Experimental procedure*

The method is based on the modification of the method explained by Jimenez *et al.* [11]. Briefly, blood was obtained from ether-anaesthetized rats and quickly centrifuged (3000 rpm, 10 min, 22 ºC). The erythrocytes were washed three times with saline (0.9%)

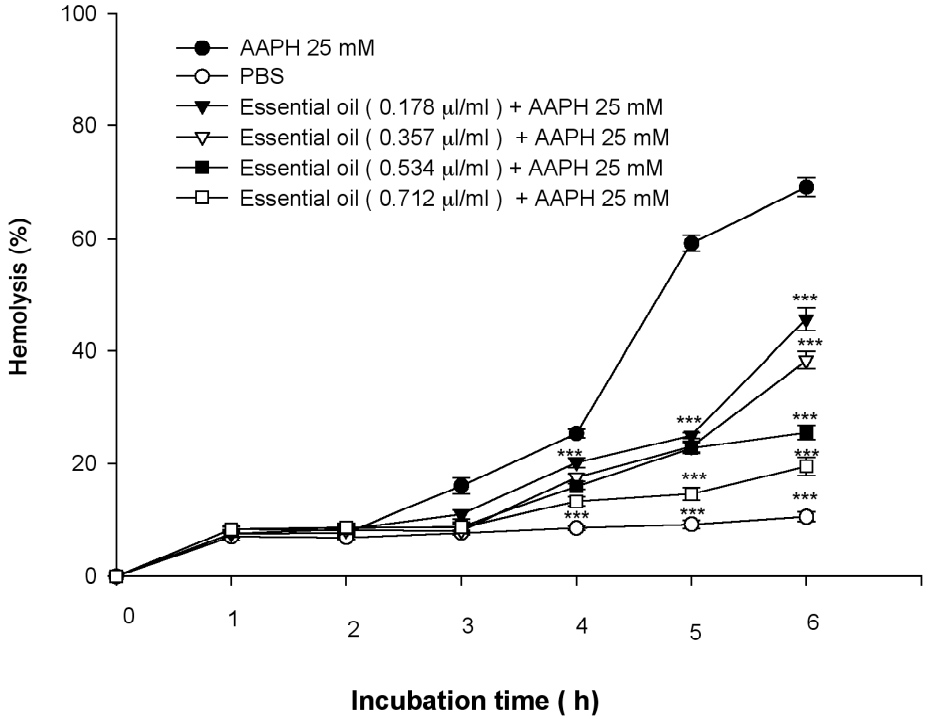


**Figure 2.** Protective effect of different concentrations of rosemary essential oil on the AAPH (25 mM)– induced erythrocytes lysis (n=5, mean±SEM, *** $p$<0.001 compared to PBS).

and centrifuged (3000 rpm, 10 min) to obtain a constantly packed cell volume. The latter was subsequently used to prepare a 10% v/v erythrocyte suspension with phosphate buffer (pH=7.4). Aliquots (2.5 ml) of the cell suspension were added to 25 ml flasks and incubated at 37 ºC for 10 min with constant and gentle shaking. Two final concentrations of AAPH (25 and 50 mM) which prepared in phosphate buffer and four final concentrations of essential oil (0.178, 0.357, 0.535 and 0.714 µl/ml) were added to the incubation medium. Samples of the suspensions were removed at different time points and the degree of hemolysis was assessed spectrophotometrically by reading the absorption of supernatant at 540 nm (absorption A). Similarly, the reaction mixture was treated with 0.1% triton X-100 solution to yield complete hemolysis, and the absorption of the supernatant after centrifugation was measured at 540 nm (absorption B). The percentage of hemolysis was calculated from the ratio of readings, (absorption A/ absorption B) x100.

*2.3. Statistical analysis*

Data were presented as mean±SEM. All data were analyzed using analysis of variance (ANOVA) followed by tukey-kramer test for comparison. Statistical significance was defined as $p$<0.05.

## 3. Results

Erythrocytes suspended in phosphate buffer solution and incubated at 37 ºC remained stable and little hemolysis was observed for 6 h (less than 8%). The addition of different concentrations of the essential oil to erythrocytes suspension showed no significant hemolysis compared with phosphate buffer solution. When AAPH (25 mM and 50 mM) was added to erythrocytes suspension, it induced hemolysis in a time-dependent manner and was characterized by an induction phase, during which no significant hemolysis took place, followed

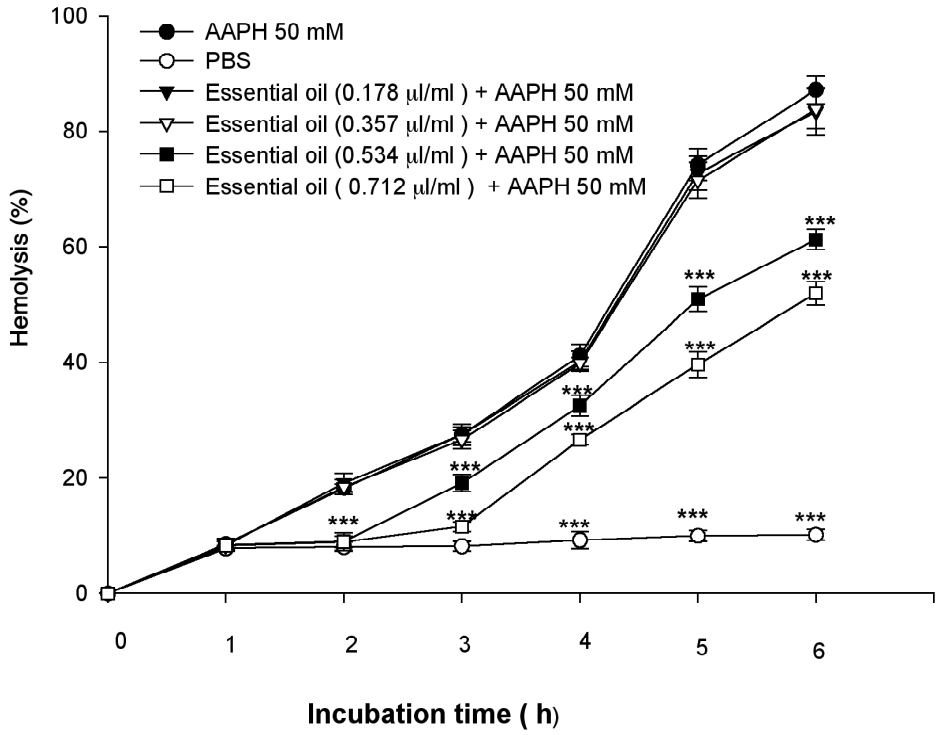


**Figure3.** Protective effect of different concentrations of rosemary essential oil on the AAPH (50 mM)– induced erythrocytes lysis (n=5, mean±SEM, *** $p$<0.001 compared to PBS).

by a sharp hemolysis phase (Figure 1). The essential oil inhibited hemolysis induced by 25 mM AAPH in a concentration-dependent manner. However, in the presence of 50 mM of AAPH, only the two higher concentrations of the essential oil (0.535 µl/ml and 0.714 µl/ml) inhibited hemolysis (Figures 2 and 3).

The effects of delayed addition of the essential oil on AAPH-induced erythrocytes lysis are shown in Figure 4. The addition of essential oil 2 h or 3 h after incubation with AAPH (25 mM) had no significant effect on the time course of cell lysis.

## 4. Discussion

Data presented here show that, in addition to its demonstrated antioxidant effects, rosemary essential oil also displays cytoprotective effects against the damage induced by AAPH to the intact erythrocytes. As rosemary has the ability to scavenge peroxyl radicals [12], its cytoprotective effects are likely to result from diminishing AAPH-derived peroxyl radicals generated during the incubation period. It is possible that during the induction period, antioxidant molecules in the essential oil are progressively consumed by AAPH–derived peroxyl radicals. Such scavenging action would lower the extracellular concentration of peroxyl radicals to a level that could be accounted for the reduction in the maximal extent of the hemolysis seen when the essential oil is added to the cell suspension.

Recent studies by Sato *et al.* [13] have shown that the hemolytic action of AAPH could be associated with the oxidation of lipid components present in the erythrocyte membranes. Thus, the possibility that rosemary exerts its cytoprotective effects at the membrane level, as a chain-breaking antioxidant, should also be considered. In fact, Miki and Mino [14] established that under experimental conditions similar to those employed here, the hemolysis ensues only after AAPH has lowered the erythrocyte

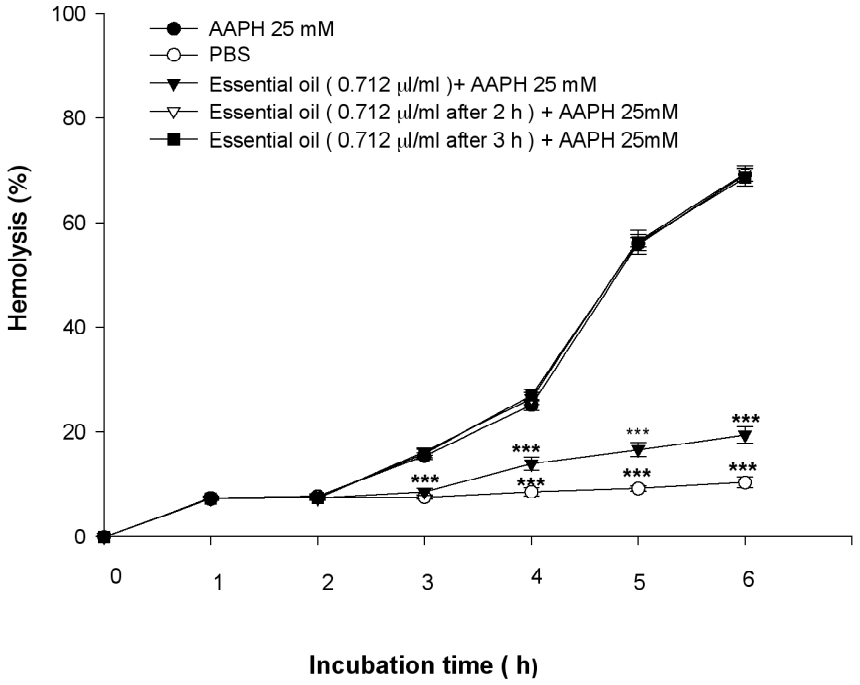


**Figure 4.** Effect of delayed addition of rosemary essential oil on the AAPH (25 mM)–induced erythrocytes lysis (n=5, mean±SEM, *** $p<0.001$ compared to PBS).

membrane α-tocopherol concentrations to a critically low level. Therefore, rosemary essential oil may interact with lipoperoxyl radicals and spare α-tocopherol molecules. Membrane protein degradation is a major event associated with the AAPH-induced lysis of erythrocytes [15]. On the other hand, rosemary may protect red blood cells lysis by preventing the oxidative damage of membrane proteins.

Our study also showed that delaying the addition of the essential oil to the incubation mixture for 2 to 3 h resulted in the loss of the effectiveness of essential oil to prevent lysis induced by AAPH. This result suggests that within this elapsed time, AAPH has already triggered oxidative modifications that can no longer be prevented or reversed by the presence of rosemary essential oil.

Since the leakage of hemoglobin into the extracellular medium represents only a final event to measure the extent of cell lysis, it is not possible from the present data to determine the mechanism(s) by which essential oil exerted its cytoprotective effects. However, given the possibility that the oxidation of membrane lipids or proteins represents a significant event underlying erythrocyte lysis, future work will address whether rosemary prevents the oxidation of both membrane components and how such effects would relate to its reported cytoprotective actions.

## Acknowledgements

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## References

[1] Halliwell B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radical Res* 1996; 25: 57-74.

[2] Slater TF, Cheeseman KH, Armstrong D, Sohal RS, Cutler RG, Proudfoot KC. *Free radicals, lipid peroxidation and cancer*. New York: Raven Press, 1984; pp. 293-305.

[3] Einsele H, Clemens MR, Remmer H. Effect of ascorbate on red blood cell lipid peroxidation. *Free Radical Res Commun* 1985; 1: 63-7.

[4] Vissers MCM, Stern A, Kuypers F, Van den Berg J, Winterbourn CC. Membrane changes associated with lysis of red blood cells by hypochlorous acid. *Free Radical Biol Med* 1994; 16: 703-12.
[5] Koga T, Moro K, Terao J. Protective effect of a vitamin E analog, phosphatidylchromanol, against oxidative hemolysis of human erythrocytes. *Lipids* 1998; 33: 589-95.
[6] Niki E, Komur, E, Takahashi M, Urano S, Ito E, Terao K. Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. *J Biol Chem* 1988; 263: 19809-14.
[7] Al-Sereti MR, Abu-Amer KM, Sen P. Pharmacology of rosemary and its therapeutic potentials. *Indian J Exp Biol* 1999; 37: 124-30.
[8] 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.
[9] Aruma OL, Halliwell B, Aeschbach R, Loligers J. Antioxidant and pro-oxidant properties of active rosemary constituents: Carnosol and carnosoic acid. *Xenobiotica* 1992; 22: 257-268.
[10] Joyeux M, Rolland A, Fleurentin J, Mortler F, Dorfman P. *tert*-butylhydroperoxide-induced injury in isolated rat hepatocytes: a model for studying antihepatotoxic crude drugs. *Planta Med* 1990; 56: 171-4.
[11] Jimenez I, Garrido A, Bannach R, Gotteland M, Speiskg H. Protective effects of blodine against free radical-induced erythrocyte lysis. *Phytother Res* 2000; 14: 339-43.
[12] Sotelo-Felix JJ, Martinez-Fong D, Muriel P, Santillan RL, Castillo D. Evaluation of effectiveness of *Rosemarinus officinalis* in the alleviation of carbon tetrachloride-induced acute hepatotoxicity in rat. *J Ethnopharmacol* 2002; 81: 145-54.
[13] Sato Y, Kamo S, Takahashi T. Suzuki Y. Mechanism of free radicals-induced hemolysis of human erythrocytes: Hemolysis by a water-soluble radical initiator. *Biochemistry* 1995; 34: 8940-9.
[14] Miki M, Mino M. Changes in RBC membrane lipids affected by two radical-producing systems and effect of α-tocopherol as a radical scavenger. *J Osaka Med Cell* 1985; 44: 60-3.
[15] Celedon G, Lips V, Alvarado C, Cortes M, Lissi E, Gonzalez G. Protein degradation in red cells exposed to 2,2'-azobis (2 amidinopropane)–derived radicals. *Biochem Mol Biol Intern* 1997; 43: 1121-7.