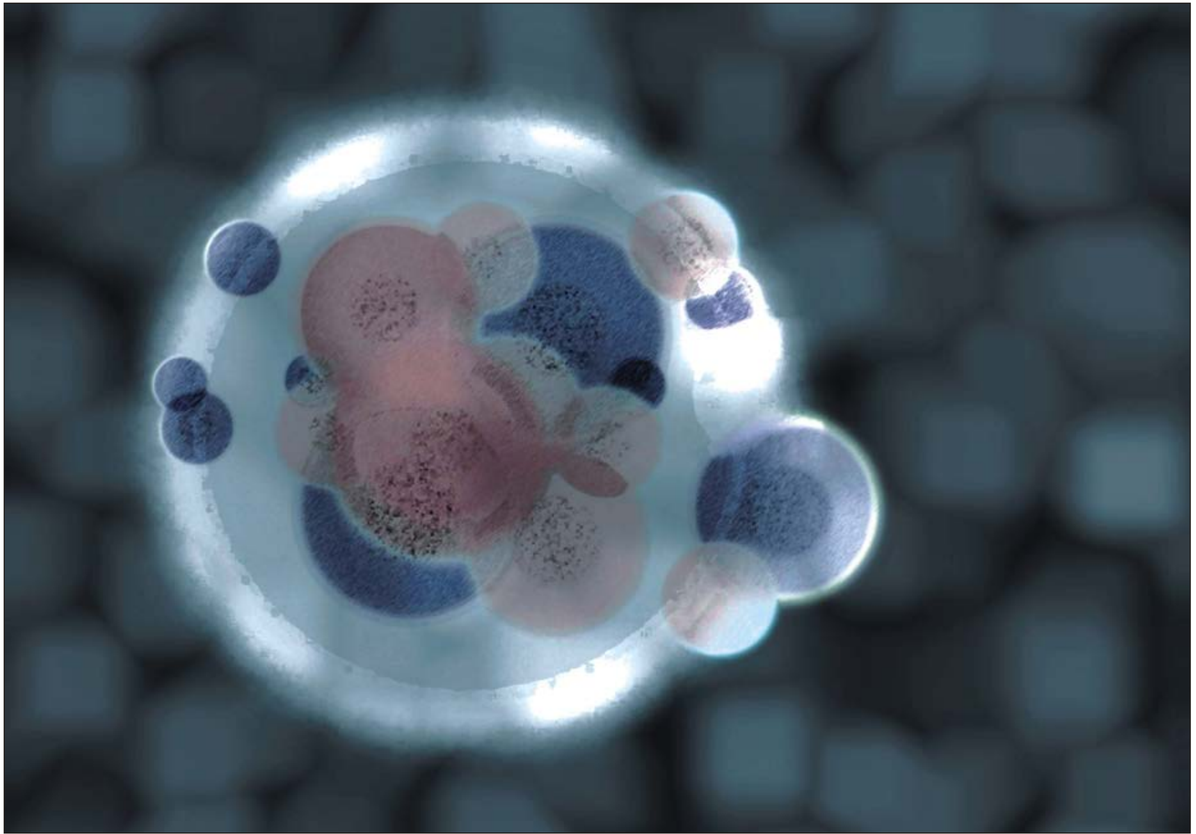

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Rosmarinic Acid Prevents the Oxidation of Low Density Lipoprotein (LDL) *In vitro*

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Abstract: The objective of the present study was to assess various antioxidative activities of Rosmarinic Acid (RA) and its effect on oxidation of Low Density Lipoprotein (LDL) induced by $CuSO_4$ *in vitro*. It was demonstrated that RA was able to inhibit LDL oxidation and decrease the resistance of LDL against oxidation. Rosmarinic acid showed a decrease the formation of malondialdehyde (MDA) rate of 31.8, 36.7 and 50.3% at concentrations ranging from 100- 400 μM, respectively, against oxidation *in vitro*. The inhibitory effects of the RA on LDL oxidation were dose-dependent. Total antioxidant capacity of RA was 0.65±0.03 mmol of ascorbic acid equivalents/mmol RA. The RA showed remarkable scavenging activity on 2,2-diphenyl-picrylhydrazyl (DPPH) (IC_{50} 0.06±0.004 μM). This study showed that RA is a potent antioxidant and prevents the oxidation of LDL *in vitro* and it may be suitable for use in food and pharmaceutical applications.

Key words: Antioxidant activity, LDL oxidation, rosmarinic acid, pharmaceutical applications, inhibitory effects

INTRODUCTION

Cardiovascular disease is one of the leading causes of mortality in our society. The increasing concentration of plasma Low Density Lipoprotein (LDL) is a major risk factor in this regard, the underlying mechanisms remain unclear and need more investigations. To date, considerable evidence supports a role for oxidatively modified LDL in the pathogenesis of atherosclerosis (Holvoet and Collen, 1998; Steinberg, 1997). The uptake of oxidized LDL (Ox-LDL) by macrophages results in the formation of foam cells and cellular cholesterol accumulated in vascular endothelial cells and promotes the development of the characteristic fatty streaks found in atherosclerotic lesions (Ani *et al*., 2007; Yoshida and Kisugi, 2010). Some diseases, such as cancer, cardiovascular diseases, diabetes, neurological disorders arthritis and inflammations are related to the imbalance of oxidants and antioxidants (Momtaz and Abdollahi, 2010). There is increasing interest in research of natural antioxidant products for use as medicines and food additives (Annegowda *et al*., 2010). Vitamin C, vitamin E and carotenoids are some of these widely used natural antioxidants. Antioxidants played an important role in lowering oxidative stresses caused by Reactive Oxygen Species (ROS). ROS including hydroxyl radical, nitroxides, superoxide anion radical and hydrogen peroxide are generated under physiological and pathological stresses in human body (Afonso *et al*., 2007). Rosmarinic acid (α-O-caffeoyl-3, 4-dihydroxyphenyl lactic acid; RA) is a naturally water-soluble polyphenolic compound. Rosmarinic acid has antioxidant (Del Bano *et al*., 2003; Hossain *et al*., 2009), antimicrobial activities (Bernardes *et al*., 2010), anti-inflammatory (Swarup *et al*., 2007), antiangiogenic (Furtado *et al*., 2010), antitumor (Osakabe *et al*., 2004) and HIV-1-inhibiting properties (Dubois *et al*., 2008). Rosmarinic acid is also used for food preservation. There is considerable experimental evidence to show that several different antioxidant compounds given at high pharmacological doses are effective in decreasing both LDL oxidation and atherogenesis in animals (Li *et al*., 2010). In humans, supplementation with antioxidants combined at physiological doses is incapable of inhibiting coronary heart disease in primary prevention (Li *et al*., 2007). Antioxidants would have to be given at high pharmacological doses in humans to inhibit ex vivo Cu^{2+}-induced LDL oxidation (Seo *et al*., 2010). Since, the inhibitory effects of RA on LDL oxidation have not previously been reported, the objectives of the present study were to assess various antioxidative activities of

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RA and investigate the effect of RA on the oxidation of LDL induced by $CuSO_4$ *in vitro* by monitoring the formation of conjugated dienes, the formation of Thiobarbituric Acid Reactive Substances (TBARS).

MATERIALS AND METHODS

Materials: Disodium ethylene diamine tetra acetate (Na_2EDTA), potassium bromide (KBr) , sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), 1,1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid, ferric chloride, sodium acetate, 2,6-di-tert-butyl-4-methyl phenol (BHT), rosmarinic acid, G, ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the solvents used were of analytical grade. The 2-thiobarbituric acid (TBA) was obtained from Fluka Chemie (Buchs SG, Switzerland).

Blood sampling: Blood samples were taken from ten men. The protocols for the blood sampling were approved by the Medical University of Lorestan Ethics Committee and all the informed constants were taken from all the men. Fasting blood samples after an overnight fasting were collected in EDTA containing tubes (1.6 mg EDTA mL blood). To obtain fresh plasma, the blood samples were centrifuged (3000 rpm for 10 min at 4°C) as soon as the samples were collected to avoid auto-oxidation. To minimize oxidation *in vitro,* sodium azide (0.06%, w/v) was added to plasma samples immediately after separation.

Isolation of LDL: The LDL fraction was isolated from fresh plasma by single vertical discontinuous density gradient ultracentrifugation (Ani *et al*., 2007; Crawford *et al*., 1999). The density of the plasma was adjusted to 1.21 g mL^{-1} by the addition of solid KBr (0.365 g mL^{-1}). Centrifuge tubes were loaded by layering 1.5 mL of density-adjusted plasma under 3.5 mL of 0.154 mol L^{-1} NaCl and centrifuged in a Beckman L7-55 ultracentrifuge at 40000 rpm at 10°C for 2.5 h. The isolated LDL was dialyzed for 48 h at 4°C against three changes of deoxygenated-PBS (0.01 mol L^{-1} Na_2HPO_4, 0.16 mol L^{-1} NaCl, pH 7.4).

DPPH free radical-scavenging activity: DPPH free radical-scavenging activity of the test samples was determined according to the method of Blois (1958). In brief, 4 mL of DPPH radical solution in ethanol (1 mM) was mixed with 1 mL of RA solution in ethanol containing 0.01-3000 μM of RA and after 30 min, the absorbance was measured at 517 nm. This activity was given as percentage DPPH scavenging that is calculated as:

$$\text{DPPH scavenging (\%)} = \frac{\text{control absorbance- RA absorbance}}{\text{control absorbance}} \times 100$$

The 50% inhibition concentration (IC_{50}), i.e., the concentration of RA that was required to scavenge 50% of radicals, was calculated. All samples were analyzed in triplicate.

Total antioxidant activity: Total antioxidant activity of the test samples was determined (Prieto *et al*., 1999; Hou *et al*., 2011). In brief, 0.3 mL of sample was mixed with 3.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95°C for 90 min under water bath. Absorbance of the samples was measured at 695 nm. The total antioxidant activity was expressed as the number of equivalents of ascorbic acid (μmol g^{-1}).

Oxidation of LDL

Continuous monitoring of formation of conjugated dienes in LDL: After isolation of total LDL, the protein content of LDL was measured (Bradford, 1976). LDL was adjusted to 150 μg mL^{-1} of LDL protein with 10 mM PBS, pH 7.4 and then aliquots of RA were added to the solution. The oxidative modification of LDL was initiated by addition of freshly prepared 10 μM $CuSO_4$ solution at 37°C in a water bath for 5 h. The kinetics of LDL oxidation was monitored every 10 min by measuring its absorbance at 234 nm. The lag phase was calculated from the oxidation profile of each LDL preparation by drawing a tangent to the slope of the propagation phase and extrapolation into intercept the initial-absorbance axis.

The lag phase represented the length of the antioxidant-protected phase during LDL oxidation by RA *in vitro*. The lag time was measured as the time period until the conjugated dienes began to increase (Navder *et al*., 1999). The formation of conjugated dienes was calculated as conjugated dienes equivalent content (nmol mg^{-1}-protein) at 5 h. The conjugated dienes concentration was calculated by using the extinction coefficient for diene conjugates at 234 nm (29500 M^{-1} cm^{-1}).

Assay of the formation of thiobarbituric acid reactive substances (TBARS): Lipid peroxidation end products were determined as TBARS according to modified method of Buege and Aust. After initiating the oxidation process with $CuSO_4$, the sample mixtures were incubated at 37°C for 5 h in a water bath and the reaction was terminated by adding EDTA (2 mM). TBARS formation was measured in a spectrophotometer at 532 nm. The results were recorded as malondialdehyde (MDA) equivalent content (nmol/mg LDL-protein) (1.56×10^5 M^{-1} cm^{-1}) (Sheu *et al*., 2003).

Statistical analysis: The data were presented as mean±SD of three experiments performed in duplicate. The variables used to describe the difference between the oxidation curves were lag time, conjugated dienes and MDA. These parameters were obtained using the Mann-Whitney test (using SPSS 13.0 statistical software) for independent data and the differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Antioxidant activity

DPPH scavenging assay: Researchers are recently interested in investigation and research into extraction of natural antioxidants from medical herbs to replace synthetic antioxidants. Natural antioxidants are healter and more beneficial and have fewer side effects than synthetic antioxidants (Rached *et al*., 2010; Prasong, 2011; Olajuyigbe and Afolayan, 2011) Phytochemicals with antioxidant effects include some cinnamic acids, rosmarinic acid, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins and triterpenes (Soobrattee *et al*., 2005; Shahbudin *et al*., 2011). Therefore, natural antioxidants such as rosmarinic acid are taken into consideration in order to inhibit diseases related to oxidative stress such as coronary heart disease, nephrotoxicity and diabetes mellitus (Tavafi *et al*., 2011; Tavafi and Ahmadvand, 2011; Hasani-Ranjbar *et al*., 2010). RA has a number of interesting biological activities, including antiviral, antibacterial, anti-inflammatory, anti allergic and antioxidant effects. Plant extracts containing RA also have excellent potential as antioxidants for food preservation (Sanbongi *et al*., 2004). Also Rosmarinic acid helps to reduce the risk of cancer and atherosclerosis. Another study demonstrated that rosmarinic acid is suitable for the treatment of rheumatoid arthritis (Al-Sereiti *et al*., 1999; Hur *et al*., 2007). Conducting research on natural antioxidants and evaluating and comparing their antioxidant effects, as well as newer and more valuable sources of natural antioxidants can be found and used in special cases.

A stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) has widely been used in the assessment of radical scavenging activity of plant extracts, natural compounds and foods (Tarawneh *et al*., 2010; Eleazu *et al*., 2011; Ramirez-Mares *et al*., 2010). The antioxidant activity of RA was evaluated by the DPPH radical scavenging capacity. Fig. 1 shows the percentage of DPPH radicals scavenging capacity with BHT as reference. In the DPPH scavenging assay, the IC_{50} (the concentration required to scavenge 50% of radical) values of RA and BHT were 0.5±0.03 and 0.06±0.004 μM, respectively. The data obtained show that RA is a free radical scavenger and may act as a primary antioxidant which can react with free radicals by donating hydrogen.

Total antioxidant activity: The phosphomolybdenum method has been widely used in the assessment of total antioxidant activity of plant extracts, natural compounds and foods. Fig. 2 shows the total antioxidant activity of ascorbic acid as standard. The total Antioxidant capacity of RA was 0.65±0.03 mmol of ascorbic acid equivalents $mmol^{-1}$ RA. Our results indicated that RA is found to posses a good antioxidant activity.

Oxidation of LDL

Continues monitoring of formation of conjugated dienes in LDL and kinetics of $CuSO_4$-induced LDL oxidation: Recently, it has become widely accepted that diet may play an important role in health promotion and disease prevention. Objective data are required by consumers and health care professionals to improve daily diets and, consequently, reduce the risk of chronic diseases such as Coronary Heart Disease (CHD) (Noroozi *et al*., 2009). Lipid peroxidation is one of principal factors in causing

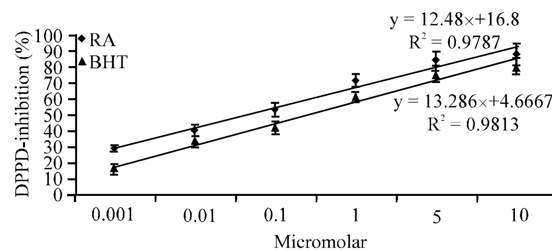

Fig. 1: Free radical scavenging capacities of the RA and BHT measured in DPPH assay. Each point represents the mean of five experiments

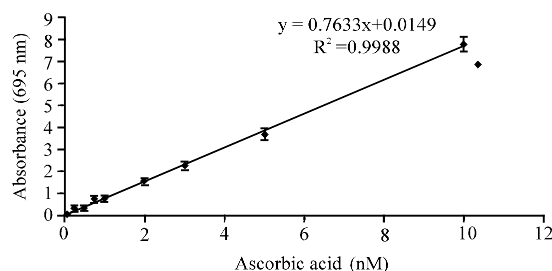

Fig. 2: Total antioxidant activity of ascorbic acid as standard measured in phosphomolybdenum method. Each point is the mean of five experiments

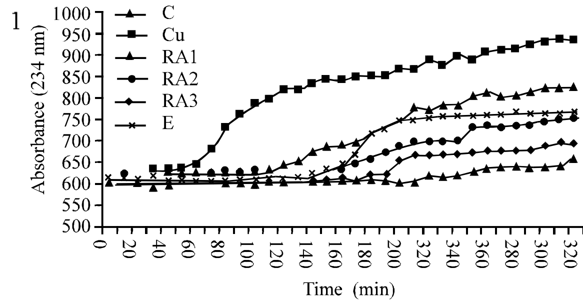

Fig. 3: The effects of RA on LDL oxidation in 10 mM PBS, pH 7.4 at 37°C for 5 h. (C) n-LDL, (Cu) n-LDL+copper, (RA1) n-LDL+RA (100 μM), (RA2) n-LDL+RA (200 μM), (RA3) n-LDL+RA (400 μM) and (E1) n-LDL+vitamin E(100 μM). Each point represents the mean of five experiments

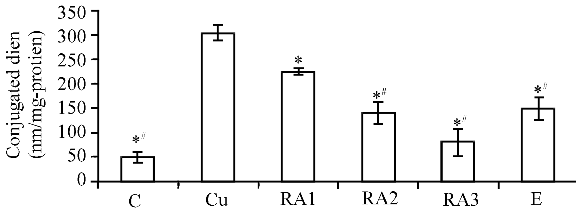

Fig. 4: The effects of RA on the formation of conjugated dienes of LDL oxidation. Each value is the mean of five experiments. *,#p<0.005, as compared with Cu and RA1 by Mann-Whitney test. (C) n-LDL, (Cu) n-LDL+copper, (RA1) n-LDL+RA (100 μM), (RA2) n-LDL+RA (200 μM), (RA3) n-LDL+RA (400 μM) and (E1) n-LDL+vitamin E (100 μM)

atherosclerosis (Steinberg, 1997). Oxidized LDL is atherogenic, it causes arterial cell death, accumulation of growth factors and cytokine release. In addition, oxidized LDL contributes to platelet aggregation, smooth muscle cell proliferation and LDL oxidation was shown in patients with hypercholesterolemia, hypertension, diabetes mellitus, chronic renal failure and in smokers (Steinberg, 1997). Thus, the consumption of natural antioxidants is beneficial in preventing atherosclerosis.

The effects of vitamin E and dose-gradient concentration of RA on the kinetics of $CuSO_4$-induced LDL oxidation are shown in Fig. 3. It shows that $CuSO_4$ dramatically increased oxidation of LDL. The formation of conjugated dienes, a marker of LDL oxidation, decreased by vitamin E and RA. Conjugated Diene (CD) formation serves as a marker of the oxidation process (Chikezie, 2011).

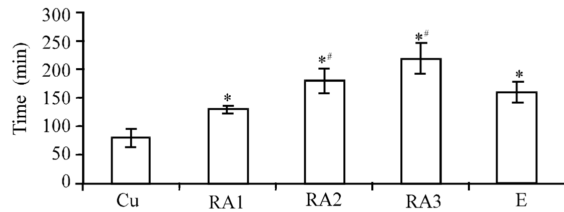

Fig. 5: The effects of RA on lag time of $CuSO_4$-induced LDL oxidation, Each point represents the mean of five experiments *,#p<0.005, as compared with Cu and RA1 by Mann-Whitney test. (C) n-LDL, (Cu) n-LDL+copper, (RA1) n-LDL+RA (100 μM), (RA2) n-LDL+RA (200 μM), (RA3) n-LDL+RA (400 μM) and (E1) n-LDL+vitamin E(100 μM)

Figure 4 shows the levels of conjugated dienes at 5 h in all the experimental groups. $CuSO_4$ increased the level of the conjugated dienes in LDL about six-fold and was significantly different from the control LDL. Vitamin E and RA (100, 200, 400 μM) inhibited the final levels of conjugated dienes in LDL oxidation (p<0.001). RA showed a dose-dependent inhibition in decreasing of conjugated dienes at 5 h. 100, 200 and 400 μM concentrations. Figure 5 shows the levels of lag time in all the experimental groups. In the assay, various concentrations of RA were confirmed to have a dose-dependent antioxidant activity by increasing lag time. 62.5% increased lag time by RA at concentration of 100 μM. At 200 and 400 μM, RA showed an increase rate of 125 and 175%. Vitamin E, as the positive control, at concentrations of 100 μM, 100% increased lag time, respectively. So RA has highly strong resistance on peroxidation. RA can provide hydroxyl to accept electrons and scavenge OH induced by $CuSO_4$. The results are considered to be noteworthy when compared to the findings of other studies concerning Antioxidant (Tepe and Sokmen, 2007). Result showed that antioxidant activity of rosmarinic acid is stronger than vitamin E.

The formation of malondialdehyde (MDA) assay: TBARS is a secondary products from lipid peroxidation in LDL. TBARS analysis measures the formation of secondary products of lipid oxidation, mainly malondialdehyde, which may contribute off-flavour to oxidized oil (Mudron *et al*., 2007). The antioxidative effect of RA on LDL was determined and expressed by measurement of MDA equivalent content. The levels of MDA after 5 h of incubation in all experiment groups are shown in Fig. 6. Vitamin E significantly inhibited MDA formation (p<0.005). RA (100, 200, 400 μM) significantly was inhibited the MDA production in LDL (p<0.005). RA

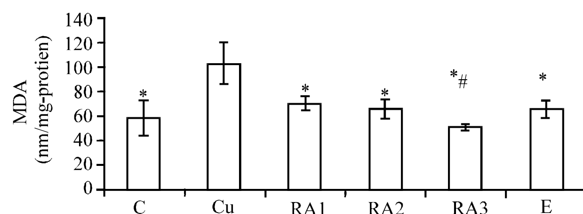

Fig. 6: The effects of RA on the formation of TBARS. Each Point is the means of five experiments

showed a dose-dependent inhibition of MDA formation at 5 h. 100, 200 and 400 μM concentration. This result suggests that RA is good antioxidants and may be used in suppressing LDL oxidation in vivo. The reduction of LDL oxidation *in vivo* may delay the progress of atherosclerosis and reduce the risk of heart diseases.

The protection of LDL by RA in a copper-induced oxidation system could be due to both metal-chelating and radical scavenging capacity. However, the mechanism by which the RA inhibits LDL oxidation *in vitro* remains unclear. Laranjinha *et al.* (1994) suggested possible explanations for the protecting effects of compounds of extracts on LDL: "(i) scavenging of various radical species in the aqueous phase, (ii) interaction with peroxyl radicals at the LDL surface, (iii) partitioning into the LDL particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals and (iv) regenerating endogenous α-tocopherol back to its active antioxidative form". The results showed that RA is a potent antioxidant and protects LDL in plasma against oxidation.

CONCLUSION

The results of the present study clearly showed that RA is found to posses a good antioxidant activity and various concentrations of RA have a dose-dependent antioxidant activity against LDL oxidation by inhibiting the formation of conjugated dienes and TBARS and increasing lag time. In conclusion, RA is a potent antioxidant and may be a good alternative to reduce the risk of atherosclerosis and coronary heart disease and other free radical associated health problems.

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