Inhibitory effect of *Allium ascalonicom* hydroalcoholic extract on low-density lipoprotein (LDL) oxidation induced by CuSO$_4$ *in vitro*

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Oxidation of low-density lipoprotein (LDL) has been strongly suggested as a key factor in the pathogenesis of atherosclerosis. Thus the inclusion of some anti-oxidant compounds in daily dietary food stuff may inhibit the production of oxidized LDL and may decrease both the development and the progression of atherosclerosis. The present work investigated the inhibitory effects of *Allium ascalonicom* hydroalcoholic extract (AAE) on LDL oxidation induced by CuSO$_4$ quantitatively *in vitro*. Oxidation of LDL was incubated with CuSO$_4$ and the formation of conjugated dienes and thiobarbituric acid reactive substances (TBARS) was assessed. It was demonstrated that AAE is able to inhibit CuSO$_4$-induced LDL oxidation. AAE showed an increase lag time rate of 11.2, 33.7 and 50.4% at concentrations ranging from 4 to 12 mg/ml, respectively, against oxidation *in vitro*. The inhibitory effects of the AAE on LDL oxidation were dose-dependent at concentrations ranging from 4 to 12 mg/ml. Total antioxidant capacity of AAE was 2.10 ± 0.20 nmol of ascorbic acid equivalents/g AAE. The AAE showed remarkable scavenging activity on 2, 2-diphenyl-picrylhydrazyl (DPPH) (IC$_{50}$ 3.68 ± 0.14 µg/ml). This study showed that AAE prevented the oxidation of LDL *in vitro* and it may suggest that they have a similar effect *in vivo*.

Key words: *Allium ascalonicom*, low-density lipoprotein oxidation, hydroalcoholic extract, antioxidants, 2, 2-diphenyl-picrylhydrazyl.

**INTRODUCTION**

Most of herbs have antioxidant properties which are active compounds such as flavonoid and phenolic compounds (Natarajan et al., 2006). Increase of free radical production and decrease of antioxidants in our body are related to the disease of induction and disease progression such as diabetes and cardiovascular diseases (Mijnhout et al., 2010). Oxidative stress involves many physiological and pathological phenomena. Because of its susceptibility to oxidation, plasma low density lipoprotein (LDL) has been used as a model to investigate oxidative damage in plasma due to exposure of LDL to CuSO$_4$-induced lipid peroxidation, reduction in atherosclerotic lesions. Although, an increasing concentration of plasma LDL is believed to be 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 (Kendler, 1987; Steinberg, 1997). It has already been shown that Alliums such as *Allium cepa* and garlic could decrease the formation of atherosclerotic lesions in animal models and epidemiological data suggest that an inverse relationship exist between the intake of antioxidant *A. cepa* and garlic and the risk of coronary artery disease (Alkreathy et al., 2010; Leopold and Loscalzo, 2009; Banerjee et al., 2002). According to the oxidation hypothesis, LDL is protected against oxidative stress by using antioxidants, thereby delaying the formation of modified LDL (Sobenin et al., 2010; Ginter and Simko, 2010; Lau, 2006).

The regular consumption of onions like garlic, has been...
shown to lower high cholesterol levels and high blood pressure, both of which may help to prevent atherosclerosis and diabetic heart disease, and reduce the risk of heart attack or stroke. Clinical trials revealed that *A. cepa* and garlic have a profound beneficial effect in the following areas: Antilipidemic, inhibition of cyclooxygenase activity and thromboxane B₂ synthesis, anticancer properties, inhibition of platelet aggregation, hydroxyl scavenging properties, antioxidant, haemostatic, and hemodynamic activities, anti-atherosclerotic, antibiotic, antiviral and antifungal activities (Alder et al., 2003; Park et al., 2008; Ostrowska et al., 2004; Nishimura et al., 2004; Krishnaswamy, 2008; Mashour et al., 1998; Sohn et al., 2009). *Allium ascalonicom* is one of the important *Allium* species commonly used in Iranian diets. Although it is widely consumed, little information is available about the beneficial effects of *A. ascalonicom* regarding its antioxidant activities. Thus this work is undertaken to investigate the effect of *A. ascalonicom* on the modification of LDL induced by CuSO₄ in vitro by monitoring the formation of conjugated dienes, the formation of thiobarbituric acid reactive substances (TBARS). Also in this study two methods were used to assess the antioxidant activity including free radical scavenging capacity and total antioxidant activity.

**MATERIALS AND METHODS**

**Chemicals**

Disodium ethylene diamine tetraacetat (Na₂EDTA), Potassium bromide (KBr), sodium chloride (NaCl), 2-2-diphenyl-picylhydrazyl (DPPH), disodium hydrogen phosphate (Na₂HPO₄) and oil red O were purchased from Sigma Chemical Co. *A. ascalonicom* was purchased from the local market.

**Preparation of A. ascalonicom hydroalcoholic extract (AAE)**

*A. ascalonicom* was weighted (0.6 g) and homogenized with 6 ml of fifty percent ethanol for 20 min. These solutions were centrifuged at 10000 x g for 10 min at 4°C and the supernatants were recovered and used at the final concentration of 4, 8 and 12 mg/ml. The *A. ascalonicom* was identified in the department of Pharmacognosy, School of Pharmacy, Jundishapour University of Medical Sciences, Ahvaz, Iran where voucher specimens were deposited (Zarei Mahmoudbadi et al., 2009).

**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, 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% wt/vol) 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; Richard et al., 1999). The density of the plasma was adjusted to 1.21 g/ml by the addition of solid KBr (0.365 g/ml). Centrifuge tubes were loaded by layering 1.5 ml of density-adjusted plasma under 3.5 ml of 0.154 mol/L NaCl, and centrifuged in a Beckman L7-55 ultracentrifuge at 40000 rpm at 10°C for 2.5 h. The yellow LDL band, located in the upper middle portion of the tube, was collected into a syringe by puncturing the tube. The isolated LDL was dialyzed for 48 h at 4°C against three changes of deoxygenated-PBS (0.01 mol/L Na₂HPO₄, 0.16 mol/L NaCl, pH 7.4) containing 0.01% NaN₃ and 0.01% EDTA.

**Oxidation of LDL**

*Continues monitoring of formation of conjugated dienes in LDL*

After isolation of total LDL, the protein content of LDL was measured (Bradford, 1979). LDL was adjusted to 150 µg/ml of LDL protein with 10 mM PBS, pH 7.4 and then aliquots of *A. ascalonicom* extracts were added to the solution. The oxidative modification of LDL was initiated by addition of freshly prepared 10 µM CuSO₄ 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 *A. ascalonicom 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-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 CuSO₄, 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° 10⁵ M⁻¹·cm⁻¹) (Hileman et al., 2004; Sheu et al., 2003).

**Antioxidant activity**

**DPPH free radical-scavenging activity**

DPPH free radical-scavenging activity of the test samples was determined according to the method of Blois (Blois, 1958). In brief, 4 ml of DPPH radical solution in ethanol (1 mM) was mixed with 1 ml of *A. ascalonicom* hydroalcoholic extract containing 0.001 to 80 µg/ml of AAE; and after 30 min, the absorbance was measured at 517 nm. This activity was given as percentage DPPH scavenging that is calculated as %DPPH scavenging=(control absorbance- AAE absorbance) / (control absorbance) x 100 The 50% inhibition concentration (IC₅₀), that is the concentration of AAE that was required to scavenge 50% of radicals, was calculated.
The effects of A. ascalonicom on LDL oxidation in 10 mM PBS, pH 7.4 at 37°C for 5 h. (C) n-LDL, (Cu) n-LDL + copper, (AAE1) n-LDL + copper + A. ascalonicom (4 mg/ml), (AAE2) n-LDL + copper + A. ascalonicom (8 mg/ml), (AAE3) n-LDL + copper + A. ascalonicom (12 mg/ml). Each point represents the mean of three experiments.

**Figure 1.**

The effects of A. ascalonicom on LDL oxidation are shown in Figure 1. It clearly shows that CuSO₄ dramatically increased oxidation of LDL. The formation of conjugated dienes, a marker of LDL oxidation, was decreased by A. ascalonicom. Figures 2 and 3 show the levels of conjugated dienes at 5 h and lag time of all the experimental groups. A. ascalonicom decreased final levels of conjugated dienes in the medium respectively. The antioxidative effect of A. ascalonicom 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 Figure 4. Addition of AAE decreased TBARS formation about 35% and was statistically significant. A. ascalonicom also decreased TBARS formation significantly.

**Total antioxidant activity**

Total antioxidant activity of the test samples was determined according to the method of Prieto et al. (1999). 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) and incubated at 95°C for 90 min under water bath. Absorbance of the samples were measured at 695 nm. Total antioxidant activity was expressed as the number of equivalents of ascorbic acid acid (µmol g⁻¹) (Prieto et al., 1999).

**Statistical analysis**

Data were presented as mean ±S.D 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 Mann Whitney test for independent data. Statistical computations were calculated using SPSS 13.0 for windows software. Differences were considered significant when p < 0.05.
The antioxidant activity of AAE was evaluated by the DPPH radical scavenging capacity. Figure 5 shows the percentage of DPPH radicals scavenging capacity with BHT as reference. In the DPPH scavenging assay, the IC₅₀ (the concentration required to scavenge 50% of radical) values of AAE and BHT were 3.68 ± 0.14 and 2.67 ± 0.11 µg/ml, respectively. Figure 6 shows the total antioxidant activity of ascorbic acid as standard. Total antioxidant activity of AAE was 2.10 ± 0.20 nmol of ascorbic acid equivalents/g AAE.

**DISCUSSION**

The oxidative modification of LDL (Ox-LDL) is the major factor that stimulates the development and progress of atherosclerosis (Steinberg, 1997). Therefore, the major objective of this study was to compare the antioxidant effects of *A. ascalonicom* using *in vitro* model. The oxidative modification of LDL induced by copper ions is shown to be related to free radical reaction, although the exact mechanism has not been elucidated yet. It is suggested that LDL oxidation may require the generation of super oxide anion and probably the ultimate generation of hydroxyl radicals by the Fenton reaction (Steinberg, 1997). After oxidation by copper ions, polyunsaturated fatty acids of LDL resulted in an elevation of lipid peroxides and depletion of vitamin E in Ox-LDL (Kendler, 1987; Steinberg, 1997; Alkreathy et al., 2010; Obata et al., 2009). Our results clearly showed that *A. ascalonicom* had antioxidant activity against LDL oxidation by inhibiting the formation of conjugated dienes and TBARS and also increasing lag time for oxidation of LDL *in vitro*. The present study indicated that *A. ascalonicom* was potent antioxidant and protected plasma LDL against oxidation. The antioxidant activity of AAE was evaluated by the DPPH radical scavenging capacity. In the DPPH scavenging
assay, the IC_{50} (the concentration required to scavenge 50% of radical) values of AAE and BHT were 8.23 ± 0.14 and 0.06 ± 0.004 µg/ml, respectively. This result showed that AAE similar to BHT, as positive control, was free radical scavengers and might act as primary antioxidant, which could react with free radicals by donating hydrogen. Also total antioxidant activity of AAE was evaluated by the phosphomolybdenum method. Total antioxidant activity of AAE was 2.10 ± 0.20 nmol of ascorbic acid equivalents/g AAE. The results of the present study indicated that AAE had good antioxidant activity. The antioxidant effect of others *Allium* species such as garlic has already been investigated and is shown to be due to the prevention of free radical oxidation (Rahman, 2001; Banerjee et al 2002; Lewin and Popov, 1994). Garlic has been used in folk medicine of many cultures for the prevention of cardiovascular diseases and other disorders (Alkreathy et al., 2010; Rahman, 2001; Banerjee et al., 2002). It has been shown in many cases that the protective effect of garlic is associated with its antioxidant properties (Lewin and Popov, 1994; http://www.nutritionj.com/sfx_links.asp?ui=1475-2891-3-10&bibl=B9Kourounakis and Rekka, 1991). Administration of *A. ascalonicom* in cyclosporine-induced nephrotoxicity in rats increased gluthathione and decreased malondialdehyde in rat kidney and protected it against oxidative damage (Wongmekiat et al., 2008). Aqueous extract of *A. ascalonicom* is also able to scavenge peroxinitrite radicals and superoxide anions (Lichtenher and Marx, 2005). The results of the present study clearly showed that AAE had good antioxidant activity and various concentrations of AAE had a dose-dependent antioxidant activity against LDL oxidation by inhibiting the formation of conjugated dienes and TBARS and increase lag time. In conclusion, AAE is a potent antioxidant and might be a good alternative to reduce the risk of atherosclerosis and coronary heart disease and other free radical associated health problems.

REFERENCES


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