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NOTE

Effects of kale (*Brassica oleracea* L. var. *acephala* DC) leaves extracts on the susceptibility of very low and low density lipoproteins to oxidation

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Of Brassicaceous plants, kale (*Brassica oleraceae* L. var. *acephala* DC) contains polyphenols, flavonoids, isoflavones and glucosinolates and so has antioxidant and anticarcinogenic properties. Antioxidants inhibit negative effects of free radicals and may, therefore, protect tissues against oxidative damage. Oxidation of lipoproteins is a key event in the development of atherosclerosis. In the current study, the levels of total phenolic and flavonoid contents and total antioxidant capacity of methanolic and aqueous extracts of kale leaves were determined. In addition, the susceptibility of isolated lipoproteins — very low density lipoprotein (VLDL) and low density lipoprotein (LDL) to the Cu^{2+}-induced oxidation with various concentrations of metanolic and aqueous extracts was evaluated as t-lag values. Although aqueous extract had higher total antioxidant capacity, methanolic extract had higher total phenolic and flavonoid content ($P<0.05$). On the other hand, both extracts inhibited lipid peroxidation in both isolated VLDL and LDL. Inhibitory effect of extracts or increasing t-lag values, mainly in methanolic extract was found to be related to increasing the concentration of extracts. It was concluded that because of high antioxidant capacity and phenolic content, kale showed a protective effect on the oxidation of lipoproteins. Therefore, it may be speculated that kale consumption may play an important protective role in the cardiovascular and other related diseases resulting from imbalance of oxidant and antioxidant status.

Keywords: *Brassica oleraceae* L. var. *acephala* DC., Kale, Antioxidants, Lipoprotein oxidation

Kale (*Brassica oleraceae* L. var. *acephala* DC.) is a leafy green vegetable belonging to the Brassicaceae family[1]. It is a low-calorie food and has a high concentration of vitamins (mainly vitamin C, E), micronutrients (iron, zinc and manganese) and macronutrients (calcium and magnesium), dietary fibre, glutamine (an amino acid with anti-inflammatory properties) and plant phytochemicals (polyphenols, flavonoids, carotenes, glucosinolates, lutein, zeaxanthin)[1-4].

The intake of natural antioxidants from foods is important for a healthy life. It is reported that the regular meals supplemented with kale juice can favorably influence serum lipid profiles and antioxidant systems[5], and hence contribute to reduce the risks of coronary artery disease in male subjects with hyperlipidemia. In case of decreased antioxidative defense and/or increased oxidative stress, lipoproteins can be oxidized and both the protein and the lipids of lipoproteins may undergo oxidative changes[6] which play important role for development of atherosclerosis and may be resulting in cardiovascular diseases. Due to a different clearance mechanism than native LDL from circulation, accumulation of oxidized LDL takes place in macrophages and smooth muscle cells, causing foam cell formation, which is thought as the initial step of the pathological process[7]. VLDL, a precursor particle of LDL, is highly susceptible to oxidation because of its high levels of triacylglycerol, cholesteryl esters and phospholipids[8]. As with small dense LDL, small dense VLDL is more susceptible to oxidative modification[9].

The role of kale in lipoprotein oxidation process has not been reported so far. Thus, in the present study, we have investigated the effects of methanolic and aqueous extracts of kale leaves on the susceptibility of LDL and VLDL to oxidation.

Materials and Methods

The chemicals were purchased from either Sigma or Merck, Germany. Fresh leaves of kale were collected and dried from five different district cities of Eastern Black Sea Region in the winter (2005). To obtain the aqeuous and methanolic extracts, dried leaves were chopped, mixed, powered by blender and extracted by using soxhlet apparatus for 24 h. The extracts were then filtered and evaporated under vacuum by using Rotary Vacuum Evaporator (R100; Bibby Sterilin Ltd., Staffordshire, UK). Dry weight of these materials was determined and stored at -20°C until use in experimental protocols.

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Determination of the antioxidant contents of kale extracts

Total phenolic content

The total phenolics were determined using the Folin-Ciocalteau reagent[10]. 100 µL of both the extracts were diluted to 3 mL with double-distilled water and 0.5 mL of Folin-Ciocalteau reagent was added. After 3 min, 2 mL of 20% sodium carbonate was added and shaken thoroughly. After 60 min of standing, the colour was measured at 650 nm (Shimadzu UV-1601 spectrophotometer) using gallic acid as standard. The results were expressed as mg equivalents/g dry weight. The within-run coefficient of variation (CV) was 7.8% (n = 10).

Total flavonoid content

Total flavonoid content was measured by the aluminum chloride colorimetric assay[11]. 1 mL of extracts, 5 mL of distilled water and 0.3 mL of 5% $NaNO_2$ were mixed. After first 5 min, 0.6 mL of 10% $AlCl_3.6H_2O$ and after second 5 min, 2 mL of 1 M NaOH was added. Finally, the volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured immediately at 510 nm. Catechin was used as standard and the results were calculated as mg catechin equivalents/g dry weight. The within-run CV was 6.1% (n = 10).

Total antioxidant capacity

Total antioxidant capacity levels were determined using automated direct measurement method[12]. Briefly, 200 µL of reagent I (acetate buffer, 0.4 mol/L, pH 5.8) was added to 5 µL samples and first absorbance A1 was readed. Then 20 µL of reagent II (the $ABTS.^+$ in acetate buffer, 30 mmol/L, pH 3.6) was added and second absorbance A2 was read after 5 min of incubation (reading point at wavelength 660 nm). Trolox was used as standard and the results were expressed as mmol Trolox/g dry weight. The within-run CV was 7.9% (n = 10).

Isolation of LDL and VLDL

Blood samples from health volunters were taken after a 12 h overnight fast and pooled plasma was separated by centrifugation (4°C, 4000 rpm, 20 min). LDL and VLDL fractions were separated by discontinuous density gradient ultracentrifugation by a modification of the method as described by Sclavons et al[13]. A discontinuous density gradient was formed in polycarbonate tubes (Beckman, 10.4 mL, lot no. 9.30-99) by adjusting density of plasma 1.30 g/mL with solid NaBr. A total of 3.5 mL of density-adjusted plasma was layered with the salt solutions (NaBr/NaCl/EDTA/NaN_3) with densities of 1.24, 1.063, 1.019 and 1.006 g/mL, respectively. Tubes loaded with the discontinuous density gradients were placed in Beckman 90 Ti fixed-angle rotor and centrifuged in a Beckman optima LE80K Ultracentrifuge at 50,000 rpm for 3 h at 4 °C. After centrifugation, VLDL (upper layer) and LDL (middle layer) were collected by using pasteur pipets. In separated VLDL and LDL, the concentrations of triacylglycerol and total cholesterol were measured by enzymatic methods using a Hitachi 917 autoanalyzer with Boehringer Mannheim (Mannheim, Germany) original reagents to check the isolation of lipoproteins. Isolated lipoproteins were dialyzed against 10 mM EDTA 10 mM PBS (pH 7.4) for 12 h to prevent oxidation and then 10 mM PBS (pH 7.4) for a further 12 h. Total protein concentration was measured method of Lowry et al[14].

Determination of susceptibility of LDL and VLDL to oxidation

Dialyzed lipoproteins (VLDL and LDL) were oxidized by Cu^{2+} [(50 µg protein of lipoprotein/mL)/(1.67 µM $CuSO_4.H_2O$) in 10 mM PBS (pH 7.4)] at 37°C without and with aqeuous or methanolic extracts in concentrations of 50, 25, 10 and 5 µg/mL, respectively. The kinetics of the oxidation of lipoproteins was determined by monitoring continuously the change in the 234 nm absorbance at 37°C using a spectrophotometer (UV-1601, UV Visible Spectrophotometer, Shimadzu) according to Kleinveld et al[15]. From the kinetic absorbance, the lag time [t(lag)] (defined as the interval between the intercept of the linear least square slope of the curve with the initial absorbance axis, expressed in min) was determined.

Stastistical analysis

Results were expressed as mean ± SD values. Significance analysis was performed by Kruskal Wallis Varyans Analyses. For pair-wise comparisions of the concentration groups in both methanolic and aqeuous extracts, Mann Whitney U Test was performed ($p<0.05$).

Results and Discussion

The antioxidant contents of kale leaves extracts are given in Table 1. Although aqueous extract had higher

Table 1—Antioxidant contents of kale (*Brassica oleracea* var. *acephala* DC) leaves extracts (n = 3)

Antioxidant parameter	Aqeuous extract	Methanolic extract
Total phenolic content (mg gallic acid/g dw)	34.3 ± 1.6	55.2 ± 4.7
Total flavonoid content (mg catechin/g dw)	6.64 ± 0.6	12.9 ± 0.9
Total antioxidant capacity (mmol/g dw)	0.24 ± 0.02	0.15 ± 0.02

Table 2—Lipid peroxidation levels of natural and oxidized lipoproteins, Cu^{2+}-induced oxidized lipoproteins without and with kale extracts at different concentrations (n = 3)

Lipoprotein types	Extracts (µg/mL)	t-lag (min) (X ± SD) 0	5	10	25	50
LDL						
Without extracts		59 ± 3.5				
With methanolic extract			65 ± 3.1	104 ± 13.9*	319 ± 18.2*	510 ± 9.2*
With aqeuous extract			60 ± 3.0	79 ± 9.2*	111 ± 12.3*	152 ± 7.2*
VLDL						
Without extract		158 ± 6.5				
With methanolic extract			218 ± 34.0*	350 ± 26.3*	636 ± 23.6*	955 ± 31.4*
With aqeuous extract			189 ± 7.8*	272 ± 22.7*	331 ± 31.7*	712 ± 13.1*

* Statistically significant ($p < 0.05$) with respect to oxidized lipoproteins without extracts

total antioxidant capacity, methanolic extract had higher total phenolic and flavonoid contents.

The effects of aqueous and methanolic extracts of kale leaves on the Cu^{2+}-induced oxidative modification of the isolated VLDL and LDL were evaluated by monitoring the kinetics of lipoprotein oxidation. From the kinetic absorbance, t(lag)s were determined and the results are shown in Table 2. It was observed that both the extracts prolonged lag time (increased the resistance of lipoproteins to oxidation) and t-lag levels increased with increasing the concentrations of extracts. Moreover, methanolic extract showed higher susceptibility than aqueous extract at the same concentration, suggesting that methanolic extract was more protective in preventing lipoproteins from oxidation. This might be because methanolic extract had higher total phenolic and flavonoid contents than aqueous extract.

Earlier, Ayaz *et al*.[16] determined nine phenolic acids of kale leaves harvested from six fields in Trabzon city in Eastern Blacksea region (the same region with our study) and found significantly higher levels of caffeic and ferrulic acids than other phenolic acids in the leaves. Caffeic acid and its methoxy-substituted derivative ferulic acid function as chain breaking antioxidants in inhibiting LDL oxidation[17]. It is reported that polyphenols bind to the LDL particle and polyphenols-enriched LDL is more resistant to oxidation than native LDL[18]. It is also reported that LDL + VLDL oxidizability is significantly decreased by the teas (which are rich in cathechins as flavonoids) and green and black tea polyphenols bind to lower density lipoproteins[19].

In this study, we also observed that the lag time for Cu^{2+}-induced oxidation of VLDL was approximately 2.5-fold longer than that of LDL (Table 2), indicating that VLDL was more resistant to oxidation than LDL. The difference between VLDL and LDL lag times in the kinetic curves was possibly due to the fact that the larger VLDL particles can contain more antioxidant molecules, such as *a*-tocopherol per lipoprotein particle than the LDL[20]. Both oxidized VLDL and LDL play a role in the development of atherosclerotic lesions[21].

In conclusion, because of high antioxidant capacity and phenolic content kale showed a protective effect on the oxidation of lipoproteins even at low concentrations. However, *In vivo* study is needed to identify the role of the eatable kale plants in the oxidation of lipoproteins and hence in cardiovascular and other diseases resulting from imbalance of oxidant and antioxidant status.

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