Food Sci. Biotechnol. Vol. 17, No. 1, pp. 151 ~ 155 (2008)

Food Science and Biotechnology

© The Korean Society of Food Science and Technology

Protection of Green Leafy Vegetable Extracts Against Oxidation of Human Low Density Lipoprotein

Cheon Ho Park[1], Oh Yun Kwon, Hyun Jung Shim, Min Hee Kim, Jeung Hee Lee, Kun Jong Lee, Xi-Wen Liu, Dai-Eun Sok[1], and Mee Ree Kim*

Department of Food and Nutrition, Chungnam National University, Daejeon 305-764, Korea
[1]College of Pharmacy, Chungnam National University, Daejeon 305-764, Korea

Abstract Oxidation of low density lipoprotein (LDL) is regarded to play an important role in the development of atherosclerosis. In the present study, salad vegetables with a remarkable DPPH radical-scavenging activity were extracted with methanol, and the methanol extracts were evaluated for the inhibition of Cu^{2+}-induced oxidation of human LDL. Separately, the amount of total phenolics was determined colorimetrically using Folin-Ciocalteu reagent. The vegetable extracts, expressing a strong inhibition of LDL oxidation (IC_{50} values, <100 μg/mL), were from angelica, dandelion, mustard leaf, and water spinach, which contained relatively high level of polyphenol content. Noteworthy, a highly positive correlation was observed between inhibition of LDL oxidation and amount of total polyphenol ($p<0.01$). Based on these results, it is suggested that salad vegetables, especially angelica, dandelion, and mustard leaf, may be used as easily accessible sources of natural antioxidants, especially in anti-atherosclerosis.

Keywords: human low density lipoprotein (LDL) oxidation, vegetable, polyphenol

Introduction

Atherosclerosis is mediated by the progressive accumulation of low density lipoprotein (LDL), within the vascular walls. Accordingly, it is generally accepted that high concentrations of LDL are strongly correlated with the development of atherosclerosis. In particular, the oxidation of LDL in artery wall was recognized as an important step in the initiation and progression of atherosclerotic lesions (1-5). When LDL is oxidized, it is modified in a variety of ways through the reaction with reactive oxygen species (ROS). The oxidation of LDL gives rise to atherogenic changes including the formation of oxidized lipids which act as chemotactic and mitogenic agents and the modification of the charge on the apolipoprotein B (apo-B) moiety of LDL creating a ligand for the scavenger receptors on macrophages (1-5). With increasing evidence that oxidized LDL is involved in atherogenesis, it is important to consume fruit and vegetables rich in antioxidative substances capable of preventing LDL oxidation (6-9). Especially, in green leafy vegetables, various kinds of antioxidants such as water-soluble as well as lipid-soluble antioxidants are contained to an appreciable level (10,11). Practically, complex mixtures of antioxidants in whole foods are responsible for their health benefits, and their advantage over single antioxidant may be due to a combination of additive and/or synergistic effects (12,13). So it is important to develop functional food materials that contain antioxidative substances in connection with the prevention of atherosclerosis. In this respect, the present study deals with the effect of total phenolics, contained in green leafy vegetables, on copper ion-induced oxidation of human LDL, and examines the relationship between the phenol content and antioxidant action against LDL oxidation.

*Corresponding author: Tel: +82-42-821-6837 ; Fax: +82-42-822-8283
E-mail: mrkim@cnu.ac.kr
Received August 29, 2007; accepted September 20, 2007

Materials and Methods

Chemicals 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E, was from Acros Organics (Morris Plains, NJ, USA). Thiobarbituric acid (TBA), trichloroacetic acid (TCA), Folin-Ciocalteu's phenol reagent, caffeic acid, tannic acid, and 1,1,3,3,-tetraethoxypropane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Ethanol, acetic acid, methanol, and hydrochloric acid were from Merck Chemical Co. (Darmstadt, Germany). All reagents used were of analytical grade.

Vegetable samples Thirteen kinds of green leafy vegetables, as listed in Table 1, were obtained from the organic farm in Gongju, Chungnam, Korea, from August of 2005 to May of 2006.

Preparation of vegetable extract Vegetables were washed, drained, weighed, and then freeze-dried. Dried samples were ground into a powder to pass through a 200 mesh sieve. Powdered samples (1 g) were immersed in absolute methanol (25 mL) and stored in the dark (15°C) for 3 days, and then the methanol fraction was collected. The extraction was repeated 3 times and solvents were removed by a rotary evaporator. Chlorophyll was removed by extracting the residue with hexane. This chlorophyll-free residue was allowed to stand at room temperature under vacuum to obtain a solvent-free powder, which was stored at −24°C. After rotary evaporation, the residue was dissolved in methanol, and the solvent fraction (mg/mL) was assayed for antioxidant activity.

Determination of total polyphenols Total polyphenol content of vegetable was determined by Folin-Ciocalteu colorimetric method (14). One mL of vegetable extract was mixed with 0.5 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water), and the mixture was allowed to stand at 22°C for 5 min; 1.0 mL of sodium bicarbonate (60 g/L) solution was added to the mixture. After 30 min at 22°C, the absorbance was measured at 760 nm using a spectrophotometer (Model 80-2088-64; Pharmacia Biotech. Co., Cambridge, England). Results are expressed as mg of tannic acid equivalents per g of the sample. Extracts were produced in triplicate and used to measure the total polyphenol content.

Isolation of human LDL Human LDL was isolated from the heparinized plasma, which was obtained from the Red Cross Blood Bank at Daejeon. LDL (1.019-1.063 g/mL) was isolated by sequential density ultracentrifugation at 4°C in an ultracentrifuge (Beckman Instruments, Mountain View, CA, USA) as described previously (15,16). The protein content was measured using the Coomassie blue reagent with bovine serum albumin as standard.

Cu^{2+}-induced oxidation of human LDL assay The antioxidant capacity of the vegetable extract was examined by evaluating the protective action of extract against copper-induced oxidation of LDL. LDL (50 μg protein/mL) was incubated with 5 μM $CuSO_4$ in 0.2 mL PBS buffer (pH 7.4) at 37°C in the absence or presence of vegetable extract for 3 hr, and then to the mixture were added 0.5 mL 20% TCA and 0.5 mL 0.68% TBA. After boiling for 15 min and cooling down in ice for 10 min, the absorbance of the supernatant after centrifugation (20,000 ×g, 5 min) was measured at 532 nm. The protective action of vegetable extract against Cu^{2+}-induced LDL oxidation was calculated according to following equation:

$$\text{Protection (\%)} = (Ac - Ax)/Ac \times 100$$

where, Ac is absorbance of positive control; Ax is absorbance of sample.

Also, The IC_{50} value reflects the concentration of vegetable extract to exert 50% inhibition of Cu^{2+} -induced oxidation of LDL. The initial background of the samples was set to 0, and the increase in absorbance of sample that consisted of LDL and $CuSO_4$, was recorded. The lag time required for the initiation of LDL oxidation was calculated from the oxidation curve. Maximal time (T_{max}), which is the time (min) required to reach the maximum level of LDL oxidation, was also determined.

Statistics All results were obtained from average of 3 independent experiments. Data were expressed as mean ±SD. The Statistical Analysis System software (17) was used to perform statistical computations. Pearson's correlation between total phenol content and antioxidant activity was performed.

Results and Discussion

Antioxidant activity of polyphenol content The generation of radical oxidative species involves radical processes generated by different biological redox systems (18,19). Therefore, the efficient removal of ROS may be crucial in preventing against lipid peroxidation of biomembranes. The partition of antioxidant compounds determines their effective antioxidant activity in either aqueous or lipid systems (19-21). The aqueous-based model, which measures the radical scavenging activity, has been used to evaluate the antioxidant activity of various vegetable extracts. Meanwhile, for the evaluation of antioxidant activity, the copper-induced oxidation of lipoprotein has been frequently employed as a model to evaluate the prevention of lipid peroxidation in a lipophilic medium. Previously, 42 kinds of vegetables, consumed frequently in Korea, were screened for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and the inhibitory effect on Fe^{2+}-induced lipid peroxidation (10,22). In the present study, the extract from 13 species of vegetables, showing high DPPH free radical scavenging activity, were taken for the determination of total polyphenol compounds. Separately, the vegetable extracts were tested for the antioxidant action against Cu^{2+}-induced oxidation of human LDL. First, when total polyphenol contents in vegetables were determined by the Folin-Ciocalteu method, the total polyphenol content of vegetables varied from 3.4 to 8.8 mg/mL (Table 1). Especially, the total polyphenol concentration is relatively high (7.3-8.8 μg/mL) in the vegetables such as angelica, dandelion, mustard leaf and water spinach. In contrast, kale, chicory, short-frui, and Lollo Rosso showed relatively low polyphenol content (3.4-4.7 mg/mL). Although high levels of total phenol in some vegetables may be due to pure polyphenol compounds, it is not excluded that the low content of water may contribute to the high amount of polyphenol compound. As presented in Table 1, the vegetables containing lower water content show higher polyphenol content. Among vegetables, the water content is the lowest (3.4 mg/mL) in angelia and dandelion, whereas the highest (8.8 mg/mL) in Lollo Rosso; the difference of total polyphenol compounds between extracts were not less than 3-fold.

Effect of vegetable extracts on protection of Cu^{2+}-induced LDL oxidation Next, we examined the antioxidant effect of vegetable extracts on Cu^{2+}-induced oxidation of human LDL. Because oxidative modification of LDL is known to play an important role in the pathogenesis of atherosclerosis and coronary heart diseases (6), and the dietary antioxidants that protect LDL from oxidation may therefore reduce atherogenesis and coronary heart diseases (19). The inhibitory effect of vegetable extract was continuously monitored during 3 hr oxidation of LDL in a system containing human LDL (50 μg protein/mL) and 5 μM $CuSO_4$ at 37°C for 3 hr, and the inhibitory effect of each extract at various concentrations was determined. Based on these, the IC_{50} value, reflecting the concentration of vegetable extract to exert 50% inhibition of Cu^{2+}-induced oxidation of LDL, was determined. As shown in Table 1, the values varied from 31 to 334 μg/mL. The extracts expressing a strong inhibition of LDL oxidation (IC_{50} value, <100 μg/mL) from were angelica, dandelion, mustard leaf (green), and water spinach. These vegetables are characterized by the relatively high content of total phenol compounds (Table 1). In contrast, the varieties such as Japanese parsley, Lollo Rosso chicory leaf

Table 1. Inhibition of human LDL oxidation (IC_{50}) and total polyphenol content of green leafy vegetable extract[1)]

Trivial name	Scientific name	Water content (%)	Extraction (%)	IC_{50} of LDL oxidation (μg/mL)	Total polyphenol content (mg/g)
Angelica	*Angelica Kiusiana* Max	84.2	22.7	31	8.8
Dandelion	*Taraxacum officinale* Wiggers	84.6	20.3	38	8.8
Mustard leaf, green	*Brassica juncea* (green)	90.8	30.3	53	8.1
Water spinach	*Ipomoea aquatica* Forsk	89.4	14.9	88	7.3
Leaf lettuce, red	*Lactuca sativa* (red)	92.0	20.3	129	5.6
Leaf lettuce, green	*Lactuca sativa* (green)	93.4	25.5	155	5.7
Chicory, witloof	*Cichorium intybus* L. (Treviso) (green)	93.2	21.8	219	5.4
Mustard leaf, red	*Brassica juncea* (red)	93.5	20.5	236	5.0
Kale, red	*Bbrassica olerecea* var. *acephala*	90.8	20.2	272	4.6
Lollo Rosso	*Lactuca sativa*	94.2	16.8	280	4.7
Japanese parsley	*Cryptotaenia japonica*	87.2	14.0	302	4.4
Kale, Scotch	*Brassica oleracea* L. var. *acephala* DC	89.8	30.3	312	3.4
Chicory, red	*Cichorium intybus* L.	90.6	22.7	334	3.8

[1)]Values are expressed as the average of triplicates.

(red), kale (red pigmented) possessing a relatively low level of phenol content (3.4-4.7 μg/mL), had a weak inhibition of LDL oxidation with IC_{50} values over 270 μg/mL. In addition, lettuce, chicon, mustard leaf (red), and kale (green) showed a modest inhibition of LDL oxidation (IC_{50}, 100-240 μg/mL) and contained an intermediate level of phenol content (5.0-5.7 μg/mL).

Inhibitory potency of angelica extract on lag phage of LDL oxidation In a separate experiment, angelica extract, showing a potent antioxidant action, were selected and further tested for the inhibitory effect on LDL oxidation in a time-dependent manner. Figure 1 demonstrates that the angelica extract prolongs the lag phase of LDL oxidation in a concentration-dependent manner.

As shown in Fig. 1, the maximal time (T_{max}) of LDL oxidation was dependent on the concentration of added vegetable extract (170-310 min for 25-100 μg/mL), and the lag time of the LDL oxidation is dependent on the vegetable extract concentration; the lag times of angelica extract are 85, 170, and 230 min for 25, 50, and 100 μg/mL of extract, respectively, compared to 65 min for control as shown in Fig. 1. Noteworthy, the inhibitory potency of angelica extract at 100 μg/mL was close to that of commercially available Trolox (1 mM). The lag phase of the LDL oxidation reaction may be affected partially by physiological lipid-soluble antioxidants such as α-tocopherol or carotenoids in natural LDL (23). Nevertheless, angelica extract expressed a potent antioxidant action against LDL oxidation at relatively low concentrations, consistent with the notion that the amount of endogenous antioxidants in LDL is limited. The delay of lag time of LDL oxidation may be related to the interference of an initial step in the development of atherosclerosis, since oxidized LDL is known to exist in atherosclerotic lesions. There are several processes for the production of oxidized LDL; one may be the oxidative modification by metal ions-induced oxidation. The oxidatively modified LDL has been shown to affect cellular functions associated with the regulation of inflammatory responses and platelet aggregation (24,25). In this respect, the intake of some vegetable extracts may be beneficial in preventing against LDL oxidation.

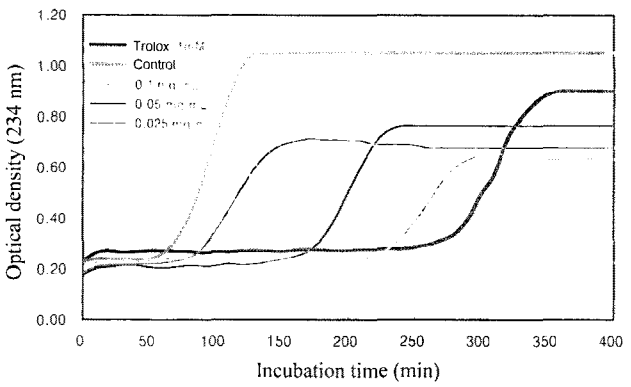

Fig. 1. Effects of angelica extract concentration on the lag time of Cu^{2+} -induced human LDL oxidation. LDL (50 μg protein/mL) was incubated with 5 μM $CuSO_4$ in 0.2 mL PBS buffer (pH 7.4) at 37°C in the presence of angelica extract of various concentrations (0.025-0.1 mg/mL), and the absorbance at 234 nm was monitored for 400 min.

Correlationship between total polyphenol content of vegetable extracts and antioxidant action In the next study, the inhibitory effect of extracts on LDL oxidation was compared to the amount of polyphenol compounds, since some vegetable extracts with great antioxidant activities in scavenging DPPH radicals contained relatively high level of polyphenol compounds in the previous study (10). As shown in Fig. 2, there was a significant positive relationship between total polyphenol content and inhibition of LDL oxidation (R^2=0.94, p<0.01).

Thus, there is a good correlationship between total polyphenol content of vegetable extracts and antioxidant action against Cu^{2+}-induced oxidation of LDL; angelica, dandelion, and mustard leaf with high total polyphenol contents showed strong antioxidant activities in copper ion-induced oxidation of LDL oxidation assay. In addition,

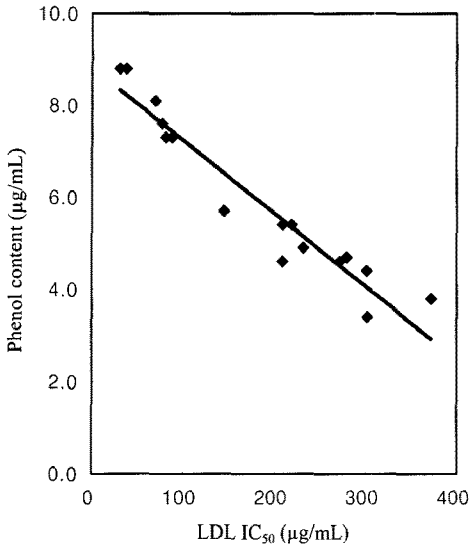

Fig. 2. Relationship between total polyphenol content and inhibition of human LDL oxidation of vegetable extracts. IC_{50} value was expressed as the concentration of each extract to express 50% inhibition of Cu^{2+}-induced LDL oxidation. Results are expressed as mg of tannic acid equivalents per g of the sample. Extracts were produced in triplicate and used to measure the total polyphenol content.

water spinach which had high polyphenol content was also so effective in preventing copper ion-induced oxidation of LDL. Thus, it is suggested that a part of antioxidant activities of extracts in copper ion-induced oxidation of LDL may be due to polyphenol compounds. The antioxidant activities of vegetables seem to be due to the effective hydrogen donor activity of polyphenol compounds (26).

In support of the above, the polyphenol compounds such as chlorogenic acid, caffeic acid, quercetin, and protocatechulic acid are considered to be principal compounds in angelica, lettuce, dandelion, or water spinach (27,28). Taken together, it is proposed that the polyphenol compounds may be responsible mainly for antioxidant action of vegetable extracts in preventing against copper ions-catalyzed oxidation of lipid as well as DPPH radical scavenging activity (29). However, it is not excluded that other vegetable components showing different antioxidant mechanisms may be implicated in antioxidant action against LDL oxidation. For example, some part of their antioxidant action may be due to the presence of antioxidant compounds, such as thiols or carotenoids, in addition to copper ion-complexing compounds. Noteworthy, the extracts from vegetables such as angelica, dandelion, mustard leaf, water spinach, or lettuce were efficient in preventing the oxidations implicated in the initial stage of atherosclerosis. Based on these findings, these vegetable extracts could be used as potential sources of natural antioxidants in anti-atherosclerosis as had been proposed for the beneficial role of polyphenols in the prevention of some diseases (30-32).

Further studies employing active antioxidant components would pose a value as a functional food material of these vegetable extracts.

Table 2. Lag time of vegetable extract (25 μg/mL) for Cu^{2+}-induced human LDL oxidation

English name	Scientific name	Lag time (min)	Maximal time (min)
Angelica	*Angelica kuisiana*	85	170
Beet leaf	*Beta vulgaris*	80	140
Dandelion	*Taraxacum officinalle wiggers*	125	170
Onion	*Allium cepa*	70	150
Control		65	130
Trolox (1 mM)		275	360

Acknowledgments

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2005-204-C00105).

References

1. Kannel WB, Castelli WP, Gordon T, McNamara PM. Serum cholesterol, lipoproteins, and the risk of coronary heart disease. The Framingham study. Ann. Intern. Med. 74: 1-12 (1971)
2. Brown MS, Goldstein JL. Lipoprotein metabolism in the macrophage: Implications for cholesterol deposition in atherosclerosis. Annu. Rev. Biochem. 52: 223-261 (1983)
3. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: An overview. Free Radical Bio. Med. 28: 1815-1826 (2000)
4. Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Radical Bio. Med. 20: 707-727 (1996)
5. Lougheed M, Steinbrecher UP. Mechanism of uptake of copper-oxidized low density lipoprotein in macrophages is dependent on its extent of oxidation. J. Biol. Chem. 271: 11798-11805 (1996)
6. Steinberg D. Antioxidants in the prevention of human atherosclerosis. Circulation 85: 2337-2344 (1992)
7. Liu S, Manson JE, Lee IM, Cole SR, Hennekens CH, Willett WC, Buring JE. Fruit and vegetable intake and risk of cardiovascular disease: The women's health study. Am. J. Clin. Nutr. 72: 922-928 (2000)
8. Kaliora AC, Dedoussis GV, Schmidt H. Dietary antioxidants in preventing atherogenesis. Atherosclerosis 187: 1-17 (2006)
9. Ryu BH. Inhibition of human low density lipoprotein oxidation by extracts from *Rhusverniciflua* strokes. Food Sci. Biotechnol. 9: 204-208 (2000)
10. Zao Xin, Song KB, Kim MR. Antioxidant activity of salad vegetables grown in Korea. J. Food Sci. Nutr. 9: 289-294 (2004)
11. Kang KH, Kwon HJ. Possible oxidation promoting activity of plant extracts with reported antioxidant activities. Food Sci. Biotechnol. 8: 97-102 (1999)
12. Eberhardt MV, Lee CY, Liu RH. Antioxidant activity of fresh apples. Nature 405: 904-905 (2000)
13. Liu RH. Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. Am. J. Clin. Nutr. 78: 517S-520S (2003)
14. Singleton VL, Rossi JA Jr. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Viticult. 16: 144-158 (1965)
15. Havel RJ, Eden HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34: 1345-1353 (1955)
16. Schumaker VN, Puppione DL. Sequential flotation ultracentrifugation. Method Enzymol. 128: 155-170 (1986)
17. SAS Institute Inc. SAS User's Guide. Statistics version 6.12. Statistical Analysis Systems Institute, Cary, NC, USA (1997)
18. Jacob RA. The integrated antioxidant system. Nutr. Res. 5: 755-766 (1995)

19. Krinsky NI. Mechanisms of action of biological antioxidants. P. Soc. Exp. Biol. Med. 200: 248-254 (1992)
20. Lee EJ, Kwon YI, Shetty K, Jang HD. Antioxidant activity of *Phodiola rosea* extracts on human low-density lipoprotein oxidation and DNA strand scission. Food Sci. Biotechnol. 13: 814-820 (2004)
21. Park YK, Lee WY, Park SY, Ahn JK, Han MS. Antioxidant activity and total phenolic content of *Callistemon citrinus* extracts. Food Sci. Biotechnol. 14: 212-215 (2005)
22. Ryu BH. Antioxidative activity of flavonoid on oxidation of human low density lipoprotein induced by macrophages and copper. Food Sci. Biotechnol. 11: 84-88 (2002)
23. Pearson DA, Tan CH, German JB, Davis PA, Gerschwin ME. Apple juice inhibits human low density lipoprotein oxidation. Life Sci. 64: 1913-1930 (1999)
24. Aviram M. Modified forms of low density lipoprotein affect platelet aggregation *in vitro*. Thromb. Res. 53: 561-567 (1989)
25. Kugiyama K, Sakamoto T, Misumi I, Sugiyama S, Ohgushi M, Ogawa H, Horiguchi M, Yasue H. Transferable lipids in oxidized low-density lipoprotein stimulate plasminogen activator inhibitor-1 and inhibit tissue-type plasminogen activator release from endothelial cells. Circ. Res. 73: 335-343 (1993)
26. Benavente-Garcia O, Castillo J, Marin FR, Ortuno A, Rio JAD. Use and properties of citrus flavonoids. J. Agr. Food Chem. 45: 4505-4515 (1997)
27. Wang SY, Chang HN, Lin KT, Lo CP, Yang NS, Shyur LF. Antioxidant properties and phytochemical characteristics of extracts from *Lactuca indica*. J. Agr. Food Chem. 51: 1506-1512 (2003)
28. Makino T, Ono T, Muso E, Honda G. Inhibitory effect of *Perilla frutescens* and its phenolic constituents on cultured murine mesangial cell proliferation. Planta Med. 64: 541-545 (1998)
29. Teissedre PL, Frankel EN, Waterhouse AL, Peleg H, German JB. Inhibition of *in vitro* human LDL oxidation by phenolic antioxidants from grapes and wines. J. Sci. Food Agr. 70: 55-61 (1996)
30. Aherne SA, O'Brien NM. Dietary flavonols: Chemistry, food content, and metabolism. Nutrition 18: 75-81 (2002)
31. Fuhrman B, Aviram M. Flavonoids protect LDL from oxidation and attenuate atherosclerosis. Curr. Opin. Lipidol. 12: 41-48 (2001)
32. Aviram M, Fuhrman B. Polyphenolic flavonoids inhibit macrophage-mediated oxidation of LDL and attenuate atherogenesis. Atherosclerosis 137: S45-S50 (1998)