Influence of Some Culinary Plants on the Suppression of Human Low Density Lipoprotein (LDL) Peroxidation (In-vitro)

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ABSTRACT
Cardiovascular disease is one of the most reasons of mortality in world. Oxidation of low-density lipoprotein (LDL) has been strongly implicated in the pathogenesis of atherosclerosis. The use of some natural antioxidant and herbs may lead to the inhibition of production of oxidized LDL and may decrease both the development and the progression of atherosclerosis. The antioxidant activities using different assays (DPPH, Xanthine Oxidase system, LDL oxidation and protein modification) in fifteen different plant parts used in culinary and herbal medicine were evaluated. Seeds of (dill, fennel, basil, turnip) and leaves of (basil, olive, jew’s mallow) showed the highest activity against inhibition of DPPH radical. Most plant extracts show relatively high activity against superoxide anion in Xanthine-XOD system. Seeds of (dill, fennel, jew’s mallow), leaves of (dill, basil, salad rocket, jew’s mallow) and thyme plant have the highest antioxidant activity against Cupper-induced oxidation of LDL. Leaves of dill, basil, olive, salad rocket, jew’s mallow and seeds of salad rocket, jew’s mallow, beside de-oiled seeds of olive can decrease the level of protein modification.
It could be concluded that culinary and medicinal herbal extracts investigated in the present study may play a role in scavenging the free radicals and increase the inhibitory effect not only against LDL lipid peroxidation, but also against modification of the protein moiety in LDL protein core, which are the early manifestations of atherosclerosis.

Keywords: Antioxidant, Edible plants, LDL peroxidation, Protein modification.

INTRODUCTION
Cardiovascular disease is one of the most reasons of mortality in world. Although an increased concentration of plasma low density lipoprotein (LDL) is believed to be a major risk factor in this regard the underlying mechanisms remain unclear and needs more investigations. Several reports have indicated the role of oxidized LDL (Ox-LDL) in the pathogenesis of atherosclerosis. uptake of Ox-LDL by macrophages results in the formation of foam cells and Ox-LDL accumulated in vascular endothelial cells, and promotes the development of the characteristic fatty streaks found in atherosclerotic lesions. Also Ox-LDL has been detected in human and animal atherosclerotic lesions. Oxidized LDL has also been reported to compromise endothelial integrity, a silent feature of atherosclerosis.

Oxidative stress describes a set of intracellular or extracellular conditions that lead to the chemical or metabolic generation of reactive oxygen/nitrogen species, including superoxide radicals, hydrogen peroxide (H2O2), hydroxyl radicals, singlet oxygen, lipid hydroperoxides, peroxynitrite, and related species. Reactive oxygen/nitrogen species can cause oxidative damage to essential cellular constituents, such as membrane lipids, proteins and DNA, which may ultimately result in cell death.

Aerobic organisms attempt to protect themselves against oxidative damage with exogenous antioxidants (e.g., vitamin E, ascorbic acid, and carotenoids) obtained through the diet as well as endogenous antioxidants (e.g., glutathione, glutathione peroxidase, catalase, and superoxide dismutase). Furthermore, oxidative damage mediated by the reactive oxygen species listed above is known to contribute to the aging process and the pathogenesis of cancer, cardiovascular disease, and other degenerative diseases.

Polyphenolic compounds are commonly found in both edible and inedible plants, they have multiple applications in food, cosmetic and pharmaceutical industries. The antioxidant capacity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators. In addition to their roles as antioxidants, these compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-inflammatory, antimicrobial, anti-thrombotic, cardioprotective and vasodilator effects. Phytochemicals, including phenolics are suggested to be the major bioactive compounds contributing to the health benefits of vegetables and fruits. It was shown that the health properties of these natural products depend on the contents of bioactive compounds, mainly phenolic compounds, and partly on dietary fibers.

Researchers are recently interested in natural antioxidants from medicinal herbs to replace synthetic antioxidants. Therefore, the research into the determination of the natural antioxidant source is very important to promote public health. Recently, natural food and food-derived antioxidants such as phenolic phytochemicals have received considerable attention, because they are as chemo-preventive agents against oxidative damages. The quest for plants with medicinal properties continues to receive attention for a
complete range of biological activities. Currently, large and ever expanding global population prefers the use of natural products in treating and preventing medical problems.21

The aim of this study was to investigate some medicinal and culinary herbs and spices which can be used in the production of phytochemicals or may be served as raw material in food and drug industry. The ethanol, 70% ethanol and water extracts were investigated for antioxidant activity by DPPH, superoxide anion radical generated in Xanthine-Xanthine Oxidase system, inhibition of LDL peroxidation and protein modification in-vitro.

MATERIALS AND METHODS

Plants processing

Raw medicinal plants used locally in Egypt, fruits, seeds, vegetables, spices were selected. Common names, scientific names and parts used are shown in Table 1. One gram of powdered sample from each plant part was extracted with ethanol, 70% ethanol and water for 24 hours to give extract 1, 2 and 3 respectively. Extracts were filtered and stored at 0°C until used.

Determination of DPPH radical Scavenging activity

The DPPH (1,1-diphenyle-2-picryl-hydrazyl) radical scavenging activity was determined according to the method of Matsushige et al.22 The absorbance was measured at 520 nm. Samples and DPPH were dissolved in methanol. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

Determination of superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity by generating superoxide anion free radical in Xanthine-Xanthine Oxidase system was measured following the method of Matsushige, et al.23 The color obtained was measured at 560 nm. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

Measurement of Copper-Induced LDL oxidation in-vitro

Isolation of LDL

LDL was isolated according to the method of Gugliucci and Menini.23 LDL (1.019-1.055 g/ml) was separated by sequential ultra-centrifugation using TL-100 Ultracentrifuge (Beckman, U.S.A.) from plasma previously added with EDTA (0.1%). LDL then extensively dialyzed against three changes of phosphate-Buffered Saline (PBS) [10 mM Sodium phosphate buffer, PH 7.2, Containing 150 mMNaCl] Containing 0.01% EDTA at 4°C. Samples were stored at 4°C in the dark and used within 24hrs. Protein Content was determined according to Lowry’s method.24 LDL was oxidized using 5µM/ml CuSO4 (freshly prepared). Incubation was carried out at 37°C for 1hr with the antioxidant (plant extract) before the addition of CuSO4.

Oxidation of LDL was monitored in the presence or the absence of antioxidant by measuring the TBARS and protein modification.

Thiobarbituric Acid Reactive Substance (TBARS) assay

LDL was oxidized using 5 µM CuSO4.25 Oxidation of LDL was monitored in the presence or absence of plant samples by measuring the absorbance of thiobarbituric acid reactive substances (TBARSs) at 534 nm using a UV spectrophotometer (UNICAM UV300). Malondialdehyde-bis-(dimethylacetal), which yields malondialdehyde (MDA) by acid treatment, was used as a standard. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

Protein Modification analysis

It was done according to the method of Visioliet al.26 LDL (200 µg protein /ml) was incubated with 5µM CuSO4 at 37°C. After 2 hours incubation was stopped. 4-hydroxynonenal–lysine and malondialdehyde-lysine adducts formation was analyzed by measuring fluorescence at E430 and E410 respectively using Spectrofluorimeter (FP-777 Jasco, Japan). Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

RESULTS

Fifteen different medicinal herbal and edible plant parts were extracted with three different systems (ethanol absolute, 70%ethanol and water).These extracts were assessed with four different procedures for antioxidant activity by measuring the DPPH radical scavenging assay, the Xanthine-Xanthine Oxidase system, inhibition of LDL peroxidation and protein modification in-vitro.

DPPH-free radical scavenging activity

The free radical scavenging activity (FRSA) of the whole plants extracts were estimated by reactivity with DPPH. From Table 1, it could be observed that: with ethanol extraction only two extracts give the highest activity; B1 (63.7%) and O1 (69.2%). Three extracts (F1, H1, J1) show moderate activity ranging from (48.6 -50.9%). With 70% ethanol extraction, six plants (A2, B2, E2, F2, D2, J2) show high FRSA ranging from (71.1 -85.9%). Five extracts show high activity (C2, G2, I2, L2, O2) ranging from (43.9 -59.1%). While with water extraction, eight plants (A3, C3, D3, E3, F3, G3, J3, O3) show high FRSA ranging from (62.4 -82.6%) and only two extracts (B3 and I3) show moderate activity (51.6 and 50.9%).

Scavenging ability for superoxide anion radical

The FRS activity of different plant extracts on superoxide anion generated by Xanthine-Xanthine oxidase enzymatic method was evaluated. It could be observed that: with ethanol extraction, twelve extracts show high FRSA ranging from (60.9%-79.9%). In 70% ethanol extraction, also twelve extracts exhibit high FRSA ranging from (65.3%-84.7%). With water extraction, thirteen extracts (A3, B3, C3, D3, E3, F3, H3, I3, J3, L3, M3, N3, O3) show
high FRSA that ranges between (61.9 - 81.3%). It is clear that most of the extracts exhibit high FRSA against superoxide anion in Xanthine-XOD system.

De-oiled seeds of turnip (H) showed higher FRS activity in DPPH and XOD assays than that of its seeds (G), while that is in contrary with De-oiled seeds of Salad rocket (K) and its seeds (L).

### Table 1: Assessment of Antioxidant activity through inhibition of DPPH and Superoxide anion radicals

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Common name</th>
<th>Part used</th>
<th>Extract (1) % inhibition of DPPH radical</th>
<th>Extract (2) % inhibition of superoxide anion radical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anethum graveolens</td>
<td>Dill</td>
<td>A - (Seed)</td>
<td>34.9 ± 0.002</td>
<td>85.9 ± 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B - (Leaves)</td>
<td>63.7 ± 0.002</td>
<td>75.3 ± 0.002</td>
</tr>
<tr>
<td>Foeniculum vulgare</td>
<td>fennel</td>
<td>C - (Seed)</td>
<td>0.60 ± 0.003</td>
<td>44.1 ± 0.002</td>
</tr>
<tr>
<td>Ocimum Basilicum L.</td>
<td>Basil</td>
<td>D - (Seed)</td>
<td>31.8 ± 0.002</td>
<td>78.6 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E - (Leaves)</td>
<td>37.3 ± 0.002</td>
<td>71.1± 0.001</td>
</tr>
<tr>
<td>Thymus vulgaris</td>
<td>Thyme</td>
<td>F - (Plant)</td>
<td>50.9 ± 0.002</td>
<td>77.2 ± 0.002</td>
</tr>
<tr>
<td>Brassica rapa</td>
<td>Turnip</td>
<td>G - (Seed)</td>
<td>4.7 ± 0.004</td>
<td>55.8± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H - (De-oiled seeds)</td>
<td>48.6 ± 0.002</td>
<td>37.2± 0.003</td>
</tr>
<tr>
<td>Olea europaea L.</td>
<td>olives</td>
<td>I - (De-oiled seeds)</td>
<td>28.7 ± 0.001</td>
<td>51.6 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>J - (Leaves )</td>
<td>49.9 ± 0.001</td>
<td>75.9 ± 0.002</td>
</tr>
<tr>
<td>Eruca sativa</td>
<td>Salad rocket</td>
<td>K - (De-oiled seeds)</td>
<td>16.0 ± 0.003</td>
<td>38.2 ± 0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L - (Seed)</td>
<td>9.1 ± 0.002</td>
<td>43.9 ± 0.001</td>
</tr>
<tr>
<td>Corchorus olitorius L.</td>
<td>Jew’s mallow</td>
<td>N - (Seed)</td>
<td>16.8 ± 0.001</td>
<td>25.8 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>O - (Leaves)</td>
<td>69.2 ± 0.002</td>
<td>59.1 ± 0.002</td>
</tr>
</tbody>
</table>

**Figure 1:** Antioxidant activity of plants (extract 1) on copper-induced human LDL peroxidation in vitro. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

**Figure 2:** Antioxidant activity of plants (extract 2) on copper-induced human LDL peroxidation in vitro. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

**Figure 3:** Antioxidant activity of plants (extract 3) on copper-induced human LDL peroxidation in vitro. Values are expressed as mean ± SD, n = 3 at a concentration of (100 µg/ml for all tested extracts).

**Figure 4:** Effect of different plant extracts on protein modification (inhibition of 4-hydroxynonenal-lysine adduct formation was analyzed by measuring fluorescence at $E_{\text{ex}}$360-$E_{\text{em}}$430).
Effect of different plant extracts on Cupper-induced oxidation of human LDL

Plant extracts showed the highest FRS activity against DPPH radical and/or Xanthine –XOD system were assessed by measuring the inhibition of human LDL oxidation in-vitro.

Effect of different plant extracts on TBARS formation

LDL oxidation was evaluated by measuring the inhibition of Thiobarbituric acid Reactive Substances (TBARS) formation. Figures 1, 2 and 3 illustrate the effect of different extracts on LDL oxidation in-vitro. In Figure 1 most of plant extracts showed relatively high protective effect against LDL oxidation in regard to oxidized LDL. Although, we cannot neglect the effect of alcohol, which causes the oxidation of normal LDL to some extent. Figure 1 showed that these plant extracts are capable to prevent oxidation of LDL due to the effect of both alcohol and CuSO₄, (A1, D1, H1, I1, M1, N1, O1).

Figure 2, shows the effect of 70% ethanol extraction on LDL oxidation. With regard to oxidized LDL, most extracts exhibit high protective effect against oxidation. These extracts are (A2, C2, E2, H2, I2, J2, M2, N2, O2). Figure 3, illustrates the effect of water extraction on LDL oxidation. Comparing to oxidized LDL, all the plant extracts show protective effect against LDL oxidation.

From data shown in Figures 1, 2 and 3, it was observed that the water extract of seeds of (dill, fennel, jewel’s mallow) and leaves of (dill, basil, salad rocket, jewel’s mallow) have the highest antioxidant activity against Cupper–induced oxidation of LDL.

Effect of different plant extracts on protein modification

Values of fluorescence at Ex360 – Em430 and Ex354 – Em410 increased 120 min after the addition of CuSO₄ to LDL samples indicating the formation of 4-HNE-lysine and MDA-lysine adducts, respectively. Pre-incubation of LDL with each plant extract resulted in marked reduction of the protein modification levels. Figures 4 and 5 showed that some plant extracts (G3, H3, I3, L3, M3, N3, O3) exerts marked reduction of fluorescence value (more than 50 %) less than the value of oxidized LDL. Other plant extracts (B3, D3, E3, F3, and J3) exert around 40% reduction in fluorescence values. The other plant extracts have no significant effect. De-oiled seeds of turnip (H) and its seeds (G), have high antioxidant activity against Cupper – induced oxidation of LDL and protein modification.

DISCUSSION

Cardiovascular disease is one of the leading causes of mortality in our society. To date, considerable evidence supports a role for oxidatively modified LDL in the pathogenesis of atherosclerosis. 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. There is an experimental evidence indicating that different antioxidant compounds given at high pharmacological doses are effective in decreasing both atherogenesis and LDL oxidation in animals. Natural antioxidants are healthier and more beneficial and have fewer side effects than synthetic antioxidants. Recently, it has become widely accepted that diet may play an important role in health promotion and disease prevention. Polyphenolic compounds in the diet enhance the stability of low-density lipoprotein (LDL) to oxidation, and evidence exists that LDL oxidation plays a significant role in atherosclerosis and coronary heart disease. Laranjinha et al. suggested possible explanations for the protecting effects of compounds and extracts on LDL: (i) Scavenging of various radical species in the aqueous phase, (ii) Interaction with peroxyl radicals at the LDL particle and terminating chain-reactions of lipid peroxidation by scavenging lipid radicals, and (iv) Regenerating endogenous α-tocopherol back to its active anti-oxidative form. For that, the antioxidant effects of plant products must be evaluated by different in-vitro assays to get relevant data. DPPH, Xanthine Oxidase system, LDL oxidation and protein modification are methods that commonly used for determining in-vitro antioxidant activity. DPPH measures sample’s free radical scavenging by combining both a hydrogen atom transfer and a single electron transfer reaction. The free radical scavenging activity of the whole plants extracts which were estimated by reactivity with DPPH, revealed that, seeds of (dill, fennel, basil, turnip) and leaves of (basil, olive, jewel’s mallow) showed the highest activity against inhibition of DPPH radical. Our data are in agreement with data obtained from a previous study. Superoxide anion is a reduced form of molecular oxygen created by receiving one electron. Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. Endogenously, superoxides could be produced in large amounts by various metabolic and physiological processes. The Formation of the
superoxide radical leads to a cascade formation of other reactive oxygen species in the cell, such as hydrogen peroxide, hydroxyl radical, peroxynitrite, or singlet oxygen in living systems. Superoxide radical decreases the activity of other antioxidant defense enzymes such as catalase and glutathione peroxidase.

In the present study the effects of different plant extracts on superoxide radical were determined by the Xanthine - Xanthine oxidase system and the results are shown in Table 1. Most plant extracts show relatively high FRSA against superoxide anion in Xanthine-XOD system. These data are in agreement with data obtained from a previous study.

The antioxidant activity of the plant extracts was also determined by the ability to inhibit LDL peroxidation. The lipid peroxidation products in un-oxidized LDL, oxidized LDL with Cu²⁺ in the presence or absence of extracts were assayed as thiobarbituric acid reactive substances (TBARS) and as protein modification products (fluorescence). TBARS, a marker for lipid peroxidation was measured. It was observed that TBARS levels were undetectable in control LDL, slightly rising only after 3hrs.of incubation. Incubation of LDL with Cupper Sulfate resulted in a marked elevation of the level of TBARS. After 24hrs.of incubation in the presence of Cu²⁺, level of TBARS did not further increase significantly (data not shown). Pre-incubation of LDL with any of the plant extracts resulted in marked inhibition of the formation of TBARS, where seeds of (dill, fennel, jowl’s mallow), leaves of (dill, basil, salad rocket, jowl’s mallow) and thyme plant have the highest antioxidant activity against Cupper-induced oxidation of LDL. These data are in agreement with data obtained from previous studies.

The LDL particle contains large amounts of polyunsaturated fatty acids which make this lipoprotein more prone to the oxidative degradation even in the absence of proxidants. The in-vitro oxidation of LDL by metal ions (Cu and Fe ) occurs in three phases: an initial lag phase (consumption of endogenous antioxidant ) a propagation phase (rapid oxidation of unsaturated fatty acid to lipid hydroperoxides) and a decomposition phase (hydroperoxides are converted to reactive aldehydes like malondialdehyde, 4-hydroxynonenal). These aldehydes react with lysine residues in apoB-100, resulting in oxidized LDL. The effect of different plant extracts on LDL resistance to oxidative modification was tested using the classical copper-catalyzed oxidation systems. The Cu-catalyzed LDL oxidation depends on the reduction of the metal ion probably through the reaction with endogenous lipid hydroperoxides, resulting in the production of lipid hydroperoxy radicals. The reduced copper in its turn decomposes preexisting peroxides, producing alkoxyl radicals. Therefore, the inhibition of the Cu-catalyzed oxidation represents the association of metal ions, chelation and scavenging of different free radicals.

Among the amino acid located mainly on protein surfaces is lysine, which is a primary target for ROS mediated oxidation with their readily hydrogen bonding properties. Side-chain amine groups of this amino acids react readily with carbonyl compounds derived from lipid oxidation and form Schiff bases, Michael adducts, and their cyclic products. Therefore, this amino acid is first modified during oxidation, and can remain reactive through propagation and termination stages.

One of the accepted procedures for measuring LDL resistance to in-vitro oxidation is to monitor fluorescence development (FD) during LDL oxidation. The fluorescence spectra of native LDL displays a band centered at 332nm. In our Experiment, the fluorescence developed during LDL oxidation was caused by reaction of protein amino groups (mainly lysine as discussed above) with aldehydes generated during decomposition of Peroxidized polyunsaturated fatty acids (PUFA). Detection of these aldehydes fluorescence is considered a marker for oxidation at the protein core of LDL. It represents the late stage of the oxidation process. Plants used in this study [dill, fennel, basil, thyme, turnip, olive, salad rocket, jowl’s mallow] are highly effective against LDL peroxidation , while some of them can decrease the level of protein modification [leaves of dill, basil, olive, salad rocket, jowl’s mallow and seeds of salad rocket, jowl’s mallow] beside de-oiled seeds of olive (Fig., 4 and 5), the data obtained in these study is in agreement with that of Visioli et al and Portella et al.

CONCLUSION

It could be concluded that culinary and medicinal herbs extracts used may play a role in scavenging the free radicals and increase the inhibitory effect not only against LDL lipid peroxidation, but also against modification of the protein moiety in LDL protein core. So, they could be useful in early manifestation of atherosclerosis.

Our study provides (for the first time) primary evidence suggesting that these culinary and medicinal herbs in further in-vivo studies could play an important role in inhibiting lipid peroxidation and protein modification in biological systems through their antioxidant, metal chelating and free radical scavenging activities.

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