Inhibitory effect of some local medicinal plants on in-vitro oxidative modification of low-density lipoprotein

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Background
Oxidative modification of low-density lipoprotein (LDL) has been implicated in atherogenesis. Antioxidants that prevent LDL oxidation may reduce atherosclerosis.
Methods
The antioxidant activity of 45 extracts from 15 locally used medicinal plants were studied in-vitro employing using three different systems; the DPPH (1,1-diphenyle-2-picryl-hydrazyl) radical scavenging assay, superoxide anion generated in Xanthine–Xanthine oxidase (X-XOD) system and the LDL oxidation induced by cupper ions.
Results
It was observed that the leaf extracts of olive, Jew's mallow, celery, the seed extract of celery and safflower, and ginger extracts had the highest antioxidant activity in the three assays.
Conclusion
It could be concluded that these plant extracts could play an important role in the inhibition of lipid peroxidation in biological systems through their antioxidant, metal chelating, and free radical scavenging activities.

Keywords:
antioxidant, DPPH, low-density lipoprotein, medicinal plants, thiobarbituric acid reactive substance, xanthine oxidase

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Introduction

Recent research has established the role of reactive oxygen species in the pathogenesis of certain human illnesses including cancer, aging, and atherosclerosis. Oxidation of biomolecules, including lipid peroxidation, involves a series of free-radical mediated chain reactions and is associated with several types of biological damages. Therefore, much attention has been focused on the use of antioxidants to inhibit lipid peroxidation and to protect biomolecules from damage by free radicals [1]. Antioxidants that inhibit oxidation of low-density lipoproteins (LPLs) have been considered to be potential antiatherogenic agents [2,3]. Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which directed most of the attention toward the naturally occurring antioxidants the use of which has mainly centered on the prevention of illness and the maintenance of health [4].

The oxidative modification hypothesis of atherosclerosis predicts that LDL oxidation is an early event in atherosclerosis [5]. Therefore, inhibition of LDL oxidation might be an important step in preventing atherogenesis [6].

Numerous natural compounds have been reported to inhibit oxidation of LPLs *in vitro* [7], such as *Gingko biloba* extract [8], several garlic compounds [9,10], mulberry leaf extract [11], buckwheat hall extract [12], *Cichorium intybus* root [13], piperlactam S, an alkaloid isolated from *Piper kadsura* [14], edible plant products [15], and chemical constituents of *Morinda citrifolia* fruits [6].

Thus, the aim of this study was to isolate highly effective antioxidants from locally used medicinal plants to protect LDL from copper-induced oxidation *in vitro*.

Subjects and methods

Plant processing and fractionation

Raw medicinal plants used locally in Egypt, fruits, seeds, roots, vegetables, and spices were selected. Common names, scientific names, and parts of the plant used are summarized in Table 1. Powdered samples (1 g) from each plant were extracted with methanol, 70% methanol, and water for 24 h to give extracts 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.* [16]. The absorbance was measured at 520 nm. Samples and DPPH were dissolved in methanol. The mean of three measurements of each

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Table 1 Assessment of antioxidant activity through inhibition of DPPH, superoxide anion radicals, and copper-induced low-density lipoprotein oxidation

Common name	Botanical name	Part used	% inhibition of DPPH radicals			% inhibition of superoxide anion radicals			% inhibition of copper-oxidized LDL								
			Extract 1	Extract 2	Extract 3	Extract 1	Extract 2	Extract 3	Extract 2	Extract 3	Extract 2			Extract 3			
									(TBARS)	(TBARS)	E_x 360–E_m 430	E_x 354–E_m 410	E_x 360–E_m 430	E_x 354–E_m 410	E_x 360–E_m 430	E_x 354–E_m 410	
Caffeic acid (10 µg)			49.8			35.3											
Black seed	*Nigella sativa*	Seed	0.0	22.3	44.9	–	–	54.4	–	45.1	–	–	13.2	66.3			
Black seed	*Nigella sativa*	Deoiled seed	7.4	29	35.7	4.9	7.9	7.9	–	–	–	–	–	–			
Celery	*Apium graveolens*	Seed	41	72.4	34.3	34.7	50.5	17.8	41.7	50.4	−80.9	−16.3	−31.1	7.9			
Celery	*Apium graveolens*	Leaf	54.4	46.3	43	37.6	22.7	35.6	50.5	45.1	−34.7	38.2	42.2	54.4			
Ginger	*Zingber officinale*	Rhizome	48.4	23	54	19.8	42.5	42.5	46.2	47	–	–	41.6	61.7			
Jew' mallow	*Corchorus olitorius*	Leaf	68.9	59	62.5	32.9	37.4	72	48.1	43.1	28.1	63.7	51	66.3			
Olive	*Olea europaea*	Leaf	49.8	75.3	62.5	9.9	25.7	0.0	48.1	46	–	–	19.1	49.2			
Onion	*Allium cepa*	Seed	0.0	0.0	0.0	–	–	–	–	–	–	–	–	–			
Parsley	*Petroselinum crispum*	Seed	42.4	31.8	6.7	67.3	79.6	74.8	–	–	–	–	–	–			
Parsley	*Petroselinum crispum*	Leaf	18.4	29.7	13.8	0	24.7	24.7	39.3	43.6	–	–	−12.9	29.3			
Safflower	*Carthamus tinctorius*	Flower	37.8	54.9	37	34.7	22.7	54.5	50.5	41.1	–	–	−34.2	22.2			
Salad leek	*Allium kurrat*	Seed	0.0	0.0	0.0	–	–	–	–	–	–	–	–	–			
Turmeric	*Curcuma longa*	Rhizome	41.7	42.8	10.6	0.0	0.0	9.9	–	–	–	–	–	–			
Turnip	*Brassica rapa*	Seed	17	67	43.5	3.9	0.0	5.9	–	–	–	–	–	–			
Water cress	*Eruca sativa*	Seed	15.9	37.5	35.3	0.0	0.0	0.0	–	–	–	–	–	–			

DPPH, 1,1-diphenyle-2-picryl-hydrazyl; E_m, emission; E_x, excitation; LDL, low-density lipoprotein; TBARS, thiobarbituric acid reactive substance.

sample was calculated. caffeic acid was used as a positive control [inhibitory concentration (IC_{50}) = 5.6 µmol/l].

Determination of superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity by generating superoxide anion free radicals in the xanthine–xanthine oxidase (XOD) system was measured following the method of Matsushige *et al.* [16]. The color obtained was measured at 560 nm. The mean of three measurements of each sample was calculated. Caffeic acid was used as a positive control (IC_{50} = 6.4 µmol/l).

Measurement of copper-induced low-density lipoprotein oxidation *in vitro*

Isolation of low-density lipoprotein

LDL was isolated according to the method of Gugliucci and Menini [17]. LDL (1.019–1.055 g/ml) was separated by sequential ultracentrifugation using a TL-100 ultracentrifuge (Beckman, Abbott Park, Illinois, USA) from plasma to which EDTA (0.1%) had been added previously. LDL was then extensively dialyzed against PBS (10 mmol/l sodium phosphate buffer, pH 7.2, containing 150 mmol/l NaCl) containing 0.01% EDTA at 4°C. LDL which will be used for oxidative modification by Cu^{2+} was dialyzed against a 1000-fold volume of PBS at 4°C. Samples were stored at 4°C in the dark and used within 24 h. Protein content was determined according to Lowry's method. LDL was oxidized using 5 µmol/l of $CuSO_4$. Incubation was carried out at 37°C for 1 h with the antioxidant (plant extract) before the addition of $CuSO_4$. Oxidation of LDL was monitored in the presence or the absence of antioxidant by measuring the amount of thiobarbituric acid reactive substance (TBARS) and protein modification.

Thiobarbituric acid reactive substance assay

LDL was oxidized using 5 mol/ml $CuSO_4$ [18], oxidation of LDL was monitored in the presence or absence of plant sample by measuring the thiobarbituric acid reactive substances (TBARS). The absorbance was measured at 534 nm using UV Spectrophotometer [UNICAM UV300]. Malondialdehyde-bis-(dimethylacetal), which yields malondialdehyde (MDA) by acid treatment, was used as a standard.

Protein modification analysis

It was carried out according to the method of Visioli *et al.* [19]. LDL (200 µg protein/ml) was incubated with 5 µmol/l $CuSO_4$ at 37°C. After 2 h (120 min), incubation was stopped.

4-Hydroxynonenal–lysine and MDA–lysine adduct formation was analyzed by measuring fluorescence at E_x 360–E_m 430 and E_x 354–E_m 410, respectively, using a spectrofluorometer (FP-777 Jasco, Japan).

Results

Fifteen different locally used medicinal plants were extracted with methanol–water in different proportions to produce 45 different extracts. The antioxidant activities of different extracts were assessed with three different assays: DPPH radical scavenging assay, superoxide anion generated in the xanthine–XOD system, and LDL oxidation assay.

Effect of different plant extracts on DPPH free radical scavenging activity

Table 1 summarizes the results of the free radical scavenging activity in the DPPH radical scavenging assay. The antioxidant activities in the DPPH scavenging assay ranged from 44.87 to 75.26%. Extract 2 of olive leaves showed the highest antioxidant activity, followed by extract 3 of celery seeds, extract 1 of Jew's mallow leaves, extract 2 of turnip seeds, extract 3 of olive leaves, and extract 3 of Jew's mallow leaves. Other extracts with relatively high antioxidant activities were the extract 2 of Jew's mallow leaves, extract 2 of safflower, extract 1 of celery leaves, extract 3 of ginger, extract 1 of olive leaves, extract 1 of ginger, extract 2 of celery leaves, and extract 3 of black seeds. The moderate effect ranged from 29.68 to 43.46% and the lowest activity was less than 29% (at a concentration of 100 μg/ml of the plant extract).

Effect of different plant extracts on the superoxide anion radical

The free radical scavenging activity on the superoxide anion radical generated by an enzymatic method was evaluated. The results are shown in Table 1. Fourteen different plant extracts showed the highest activities that ranged from 79.6 to 42.6%; the moderate antioxidant activities ranged from 37.4 to 22.8%. From these data it was clear that all the extracts of celery leaves and the extract 3 of Jew's mallow showed the highest activity, followed by extract 3 of black seeds, ginger, and extract 2 of parsley seeds.

Antioxidant activity of plant extracts on copper-induced low-density lipoprotein oxidation

The plants showed the highest free radical scavenging activity against the DPPH radical and/or the xanthine–XOD system, which was assessed by measuring the inhibition of human LDL oxidation *in vitro*.

TBARS, an index of lipid peroxidation, were undetectable in control LDL, with the level rising slightly only after 3 h of incubation. Incubation with the oxidant resulted in a marked elevation of TBARS. After 24 h of incubation in the presence of the oxidant, the level TBARS did not further increase significantly (data not shown). Preincubation of LDL with any of the plant extracts that showed the highest free radical scavenging activity in DPPH and/or the xanthine–XOD system resulted in significant inhibition of TBARS accumulation. From the data shown in Table 1 it was clear that extract 2 of celery, olive, Jew's mallow leaves, safflower, and ginger and extract 3 of celery seeds, ginger, and olive leaves had the highest antioxidant activities against copper-induced LDL oxidation.

Effect of different plant extracts on protein modification

Values of fluorescence at E_x 360–E_m 430 and E_x 354–E_m 410 increased 120 min after the addition of $CuSO_4$ to the LDL samples, indicating the formation of 4-hydroxynonenal–lysine and MDA–lysine adducts, respectively. Preincubation of the samples with the plant extracts markedly reduced protein modification (Table 1).

Discussion

LDL lipid peroxidation is considered to be essential in the pathogenesis of atherosclerosis [20,21]. Although data concerning the mechanisms by which lipid peroxidation occurs *in vivo* are scarce, several lines of evidence suggest that some endogenous and exogenous compounds with antioxidant activities could have some beneficial effects in the prevention of the disease. Many plant phenols and flavonoids may be important dietary antioxidants [22,23].

In this study, we set out to demonstrate the antioxidant properties of 45 different extracts using three different assays: the DPPH radical scavenging assay, superoxide anion generated in the xanthine–XOD system, and the LDL oxidation assay.

In the DPPH radical system, antioxidants directly react with the DPPH radical. In the xanthine–XOD system, a superoxide anion radical is enzymatically generated. The harmful effect of superoxide is reduced by the XOD present in the animal body. Plant extracts also showed activities similar to those of the superoxide dismutase enzyme. The activities of some plant extracts in these two systems showed similar trends; thus, in the present study, DPPH free radical (chemical) and xanthine–XOD (enzymatic) systems were selected to isolate plant extracts that have high antioxidant activities. It is believed that finding a new property in locally used medicinal plants that have withstood the test of time in terms of lack of toxicity may prove extremely relevant.

Transition metals are powerful initiators of lipid peroxidation. It was observed that several aldehydes are formed, mainly 4-hydroxy-2-nonenal and MDA [24,25]. The formation of MDA was monitored by measuring the amount of TBARS. LDL oxidation can also be detected spectroflurometrically by measuring the amount of MDA–lysine or histidine adducts formed. These protein modifications of LDLs alter their charges and configurations, leading to more negatively charged LDLs with atherogenic properties [24,25].

Antioxidants have two basic mechanisms of action, the first is by free radical scavenging (i.e. electron donation) and the second is by chelating transition metal ions [7,26,27]. In the DPPH system, some plant extracts show high free radical scavenging activities by quenching the stable free radical DPPH (Table 1). The antioxidant activity of plant extracts was tested on a different radical generated enzymatically, namely, the superoxide anion radical generated in the xanthine–XOD system (Table 1). The activities of some plant extracts in these two systems showed similar trends. From the two different systems it

was observed that plants with high antioxidant activities act by a free radical scavenging mechanism through electron donation.

The data shown in Table 1 indicate that some plant extracts have high antioxidant activities because of their ability to inhibit LDL oxidation. A mechanism suggested that these plant extracts act by metal chelation. Some of the plant extracts had the ability to protect LDL against protein modification.

Conclusion

From all of the above mentioned data it could be concluded that the leaf extracts of olive, Jew's mallow, and celery; seed extracts of celery and safflower; and ginger extracts had the highest antioxidant activities in the three assays. Thus, it was suggested that these plant extracts could play an important role in the inhibition of lipid peroxidation in biological systems through their antioxidant, metal-chelating, and free radical scavenging activities.

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Conflicts of interest

There are no conflicts of interest.

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