Phenolic-extract from argan oil (Argania spinosa L.) inhibits human low-density lipoprotein (LDL) oxidation and enhances cholesterol efflux from human THP-1 macrophages

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Received 16 December 2004; received in revised form 12 May 2005; accepted 17 May 2005
Available online 12 July 2005

Abstract
Argan oil is rich in unsaturated fatty acids, tocopherol and phenolic compounds. These protective molecules make further study of its cardiovascular diseases (CVDs) action interesting. Furthermore, no previous study has explored the antioxidant activity of argan oil in comparison with olive oil. The present study was conducted to evaluate the beneficial properties of Virgin argan oil phenolic extracts (VAO-PE) towards CVD by: (A) protecting human low-density lipoprotein, LDL against lipid peroxidation and (B) promoting high-density lipoprotein (HDL)-mediated cholesterol efflux. Human LDLs were oxidized by incubation with CuSO4 in the presence of different concentrations of VAO-PE (0–320 μg/ml). LDL lipid peroxidation was evaluated by conjugated diene and MDA formation as well as Vitamin E disappearance. Incubation of LDL with VAO-PE significantly prolonged the lag-phase and lowered the progression rate of lipid peroxidation (P < 0.01) and reduced the disappearance of Vitamin E in a concentration-dependent manner. Incubation of HDL with VAO-PE significantly increased the fluidity of the HDL phospholipidic bilayer (P = 0.0004) and HDL-mediated cholesterol efflux from THP-1 macrophages. These results suggest that Virgin argan oil provides a source of dietary phenolic antioxidants, which prevent cardiovascular diseases by inhibiting LDL-oxidation and enhancing reverse cholesterol transport. These properties increase the anti-atherogenic potential of HDL.

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Keywords: Antioxidants, Lipoproteins, Argan oil, Phenolic compounds, Reverse cholesterol transport

1. Introduction
Coronary heart disease (CHD) is the main cause of mortality in the western world [1]. Oxidation of low-density lipoproteins (LDLs) is considered an early event in the development of atherosclerosis, the underlying cause of coronary heart disease [2,3]. Oxidized LDLs are not recognized by the LDL-receptor Apo (B/E), but are taken up by arterial-wall cells, especially by macrophages, in a non-regulated manner through the “scavenger-receptor pathway”. This process leads to the formation of foam cells, the hallmark of the atherosclerosis lesion [4]. Several clinical and epidemiological studies have demonstrated an inverse association between high-density lipoproteins (HDLs) and the risk of coronary heart disease [5]. It has been established that reverse cholesterol transport (RCT) constitutes one of the main protective properties of HDL. The first and most important step of RCT is to remove excess cholesterol from cells (cholesterol efflux) [6]. Cell cholesterol efflux could be explained by different mechanisms, including aqueous diffusion, lipid-free apolipoprotein membrane microsolubilisation, SR-BI-mediated cholesterol exchange and the recently proposed role of the ABCA-1 gene [7]. All these steps are initiated by the binding of HDL to cell membrane domains. Consequently the HDL
phospholipid bilayer fluidity plays a key role in RCT mechanisms.

Dietary phenolic compounds, ubiquitous in vegetables and fruits and their juices possess antioxidant activity that may have beneficial effects on human health [8]. Recent epidemiological studies have shown that diets rich in plant-derived foods, and in phenolic compounds, are associated with a reduced incidence of cardiovascular mortality [9]. Consumption of unsaturated fatty acids (mainly oleic and linoleic acid) is associated with a reduced risk factors for cardiovascular mortality [10]. Phenolic compounds have been shown to possess free radical-scavenging and metal-chelating activities in addition to the reported anticarcinogenic properties [11]. These plant-based, non-nutrient phytochemicals may have a protective effect towards the susceptibility of LDL to oxidative modification and ultimately, to atherosclerosis [9]. Various in vitro studies using different methods of oxidation have shown that phenolic compounds from red wine [12], green tea [13], and olive oil [14] can inhibit LDL oxidation and reduce risk factors for cardiovascular disease (CVD).

Argan oil obtained from *Argania spinosa* L. seeds is eaten raw in southwest of Morocco and is also used in traditional medicine. Chemical analysis of this oil highlighted a glyceride fraction (99%) that is rich in polyunsaturated fatty acids like oleic (47.7%) and linoleic acid (29.3%) [15]. Studies with the unsaponifiable fraction revealed that argan oil is rich in tocopherol (620 mg/kg versus 320 mg/kg in olive oil) [16,17]. This fraction also contains other important compounds such as squalene, sterols (Schottenol and Spinasterol) and phenols (Ferulic, Syringic and Vanillic acid) [16,17]. These compounds make argan oil an important source of antioxidant [15], which certainly play an important role in vivo.

We have previously reported that ingestion of argan oil has an anti-hypertensive and anti-hypercholesterolemic effect [18,19]. In this work, we report the beneficial effects of phenolic extracts from Virgin argan oil (VAO-PE) in protecting human-LDL against lipid peroxidation and enhancing reverse cholesterol transport from human THP-1 macrophages.

2. Materials and methods

2.1. Chemicals

Acetic acid, sulfuric acid, sodium phosphate, thiobarbituric acid, n-butanol, methanol, ethanol, n-isopropanol and hexane were purchased from Fisher (Montreal, Que.). 1,1,3,3-Tetraethoxypropane, d-α-tocopherol, γ-tocopherol, butylated hydroxytoluene (BHT), cupric sulfate (CuSO₄), ethylenediaminetetraacetic acid (EDTA) and lithium perchlorate, DPH (1,6-diphenyl-1,3,5-hexatriene), were obtained from Sigma (St. Louis, MO). Dialysis bags were purchased from Spectrum Medical Industries (Houston, TX).

2.2. Subjects

Sera were obtained from 12 healthy volunteers (aged 20–25 years). They were all in general good health. Blood pressure, glycemia and lipid profiles were within normal ranges. The Ethics Committee of the Sherbrooke Geriatric University Institute approved the study, and all subjects gave written informed consent.

2.3. Phyto-chemistry

The phenolic compounds were extracted from argan oil according to the method of Pirisi et al. [20]. Briefly, argan oil was mixed with n-hexane and methanol/water and then stirred in a vortex apparatus and centrifuged. The hydro-alcoholic solution was washed with n-hexane and then lyophilized overnight.

2.4. Biochemical study

2.4.1. LDL and HDL isolation

Lipoprotein isolation was performed according to the method of Sattler et al. [21]. Briefly, human plasma was collected in EDTA (0.4 g/l). Isolation of lipoproteins was performed as previously described by Khalil et al. [22]. Isolated lipoproteins were dialyzed overnight at 4 °C with 10–7 M (sodium phosphate buffer, pH 7). LDL and HDL were then adjusted to a concentration of 100 and 200 μg protein/ml, respectively by dilution in the same buffer. Proteins were measured by commercial assay (Biorad, Canada).

2.4.2. Copper-mediated LDL oxidation

Peroxidative treatment of lipoproteins was carried out as previously described using transition metal ions as oxidizing agents [22]. Briefly, lipoproteins ([LDL (100 μg/ml) or (HDL 200 μg/ml)], were suspended in 10 mM in sodium phosphate buffer pH 7 and incubated with or without VAO-PE (0–320 μg/ml) for different times (0–8 h) at 37 °C in the presence of 10 μM cupric sulfate. Oxidation reactions were stopped by cooling in an ice bath after EDTA addition and the resulting lipid peroxides were measured immediately.

2.4.3. Biochemical markers of lipid peroxidation

2.4.3.1. Conjugated diene formation. LDL (100 μg/ml) oxidized alone or in the presence of various concentrations of VAO-PE (0–320 μg/ml), was continuously monitored at 234 nm to detect the formation of conjugated dienes as previously described [23].

2.4.4. Thiobarbituric acid-reactive substances (TBARS) formation

TBARS, mainly malondialdehyde (MDA), were assayed by high-performance liquid chromatography (HPLC) as described by Agarwal and Chase [24]. The column was a HP hypersil 5 μm ODS 100 mm × 4.6 mm with a 5 μm ODS guard column and the mobile phase was a methanol-buffer...
(40.60, v/v). The fluorescence detector was set at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Samples of LDL were treated with the antioxidant (BHT) and heat derivatized at 100 °C for 1 h with thiobarbituric acid at an acidic pH. Samples were extracted with n-butanol and 10 μl volumes of the extract were injected.

2.4.5. Vitamin E (α and γ-tocopherol) measurement
LDL endogenous Vitamin E was assayed as α and γ-tocopherol, at different oxidation times by reversed-phase-HPLC, electrochemical detection and UV detection at 232 nm as previously described [22]. α and γ-tocopherol were assayed on a sephasil peptide column (C18, 5 μm ST 4.6×250) (Pharmacia Biotech, Piscataway, NJ), with methanol–ethanol–isopropanol 88:24:10, v/v/v, containing lithium perchlorate (20 μM) at a flow rate of 1 ml/min.

2.4.6. Electrophoresis of LDL
The electrophoresis mobility of LDL was used as an indication of protein oxidation and was measured using agarose gel (Titan gel lipoprotein electrophoretic system). Electrophoresis was performed on 2 μl samples in barbital buffer at pH 8.6 on 0.6% agarose gels (Helena Lab., Montreal, Que.) at a constant voltage (80 V) for 45 min, then oven dried at 75 °C and stained with fat Red 7B, 0.1 in 95% methanol.

2.4.7. Fluorescence anisotropy
Lipoprotein fluidity was measured using the fluorescent probe DPH dissolved in tetrahydrofuran as described previously [25]. Briefly, lipoproteins were incubated overnight with or without VAO-PE before adding DPH (1 μM) for 30 min at 37 °C. Fluidity, represents the inverse values of anisotropy and is expressed as: I/I₀ = (1/v1)G(v2/v1−G(v2)−2v0) where I₀ and I are the parallel and perpendicular polarized fluorescence intensities and G is the monochromator grating correction factor.

2.4.8. Kinetic profile parameters of LDL oxidation
The kinetic profile of lipid peroxidation is characterized by three mathematical parameters: the lag preceding rapid oxidation denoted the lag phase, the maximal rate of oxidation (Vmax) and the maximal accumulation of oxidation products (ODmax). These three parameters were determined as previously described by Lichtenberg and Pinchuk [26].

2.4.9. Cell culture and [3H]-free cholesterol efflux measurements
THP-1 human was maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C, 5% CO2. THP-1 monocytes were incubated at 1 × 106 cells/ml in the presence of phorbol myristate acetate (PMA) for 96 h to induce differentiation into macrophages. THP-1 macrophages cells were labeled with [1α, 2α(α)-3H]cholesterol (specific radioactivity 42 Ci/mM, Sigma) for 48 h. The cells were then washed six times with serum-free RPMI medium containing 1% BSA and then equilibrated overnight under these conditions. To measure the effect of VAO-PE (320 μg/ml) on cholesterol efflux, cells were incubated for 24 h with 50 μg/ml of native HDL or oxidized-HDL (0, 4 and 8 h) with or without VAO-PE. At the end of the time course, cells were centrifuged (10,000 × g for 20 min) to remove the medium and then lysed. Aliquots (100 μl) from the medium and cells were counted separately using liquid scintillation counting. Cholesterol efflux (%), was expressed as cpm in the efflux media divided by total cpm (media plus cell) and multiplied by 100%.

2.5. Statistical analysis
Values are expressed as the mean ± S.E.M. One-way analysis of variance (ANOVA) was used for multiple comparisons. Linear regression analysis was used to assess the association between two continuous variables. Statistical analyses were performed using Prism 2.0 version software.

3. Results
The oxidation of lipoprotein is characterized by three phases: an initial lag phase followed by propagation and terminal phases. The generation of CD and TBARS during copper-initiated LDL oxidation is shown in Fig. 1A and B. In control LDL, a significant increase in CD and MDA equivalent (P < 0.001) was observed from 0 to 8 h, this increase was maximal following 8 h of oxidation. When the LDLs were oxidized in the presence of increased concentrations of VAO-PE, CD and MDA formation was significantly inhibited in a dose dependent manner. In fact, the addition of VAO-PE at concentrations ranging from 40 to 320 μg/ml increased the lag phase before conjugated diene formation in a dose dependent manner (r² = 0.9469, P < 0.001; linear regression, Fig. 1C) and MDA generation (r² = 0.9791, results not shown). At 320 μg/ml VAO-PE significantly inhibits CD and MDA formation by (88.21 and 98.7%, respectively) after 2 h of oxidation, and by (80.14 and 92.9%, respectively) after 4 h of oxidation.

Fig. 1D and E show that VAO-PE significantly reduces both the maximal rate of oxidation (Vmax) and the maximal accumulation of oxidation products (ODmax) (r² = 0.6056 and r² = 0.847, P < 0.001; respectively, linear regression). To gain more insight into the effect of VAO-PE on LDL oxidation, we measured its effect on the rate of disappearance of Vitamin E (α and γ-tocopherol). Oxidation of LDL alone resulted in a significant increase in the α- and γ-tocopherol disappearance rates (Fig. 2A and B), whereas in the presence of increased concentrations of VAO-PE the depletion of α and γ-tocopherol is significantly reduced.

To investigate the protection of VAO-PE towards electrophoretic charge modification of LDL-protein moiety (apo B) induced by oxidation was carried out on LDL oxidized...
with or without VAO-PE. Fig. 3 shows the electrophoretic mobility of LDL apo B oxidized alone or in the presence of VAO-PE. Oxidation of LDL greatly increased its electrophoretic mobility, mainly at times greater than 2 h of oxidation. This increase in mobility was abolished in the presence of 320 μg/ml VAO-PE (Fig. 3 and Table 1).

To further understand the mechanism of the phenolic compounds from VAO, we also investigated their role on membrane stabilization. The change in the relative lipid-bilayer fluidity of LDL and HDL as a function of VAO-PE concentrated was studied. Values of lipoprotein fluorescence anisotropy (r) significantly decreased when HDL and LDL were incubated overnight with VAO-PE (320 μg/ml) [control-HDL: 0.207 ± 0.005 versus VAO-PE-HDL: 0.129 ± 0.01, n = 4 (P < 0.001); control-LDL: 0.248 ± 0.003 versus VAO-PE-LDL: 0.217 ± 0.002, n = 5 (P < 0.01)]. This decrease indicates that the lipid-bilayer fluidity of HDL and LDL increased in the presence of VAO-PE (Fig. 4A and B, respectively).

HDL lipid-bilayer fluidity is an excellent marker for HDL-mediated cholesterol efflux [27]. Therefore, the effect of VAO-PE on HDL to promote cholesterol efflux was investigated. Following [3H] cholesterol preloading of THP-1 macrophages, native-HDL pre-incubated with VAO-PE increased cholesterol efflux from THP-1 macrophages (P = 0.0245, Fig. 5A). Copper-induced oxidation of HDL sig-
Effect of increasing concentrations of VAPE (0–320 μg/ml) on endogenous α-tocopherol (A) and γ-tocopherol (B) disappearance during LDL oxidation. Oxidation was induced by incubation of human LDL (100 μg/ml) with copper (10 μM). Results present mean ± S.E.M. of a minimum of three independent experiments.

Fig. 2. Electrophoretic mobility of LDL on agarose gels. Samples were electrophoresed for 45 min at 80 V and then stained with Titan gel lipoprotein stain (Fat red 7B). Lanes are identified as: (A) LDL-control, incubated with copper (10 μM) for 0, 1, 2 and 8 h. (B) LDL-treated, incubated with copper (10 μM) in the presence of 320 μg/ml of VAPE. Experiments were repeated three times and the gel shown is typical of the results obtained.

Table 1

<table>
<thead>
<tr>
<th>Oxidation time (h)</th>
<th>Control (α-tocopherol)</th>
<th>With VAPE (α-tocopherol)</th>
<th>Statistical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.5 ± 0.012</td>
<td>0.5 ± 0.009</td>
<td>ns</td>
</tr>
<tr>
<td>1</td>
<td>0.52 ± 0.012</td>
<td>0.5 ± 0.009</td>
<td>ns</td>
</tr>
<tr>
<td>2</td>
<td>1.23 ± 0.015</td>
<td>0.61 ± 0.015</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>8</td>
<td>1.79 ± 0.02</td>
<td>1.24 ± 0.006</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Data are expressed as relative electrophoretic mobility of each band at increasing oxidation time in the absence or presence of VAPE (320 μg/ml). Results are represented as the mean ± S.E.M. of three independent experiments.

Significantly reduces its capacity to recuperate [3H]-free cholesterol from THP-1 macrophages. This decrease was abolished when HDL oxidation was carried out in the presence of 320 μg/ml of VAPE (Fig. 5B).

Fig. 3. Electrophoretic mobility of LDL on agarose gels. Samples were electrophoresed for 45 min at 80 V and then stained with Titan gel lipoprotein stain (Fat red 7B). Lanes are identified as: (A) LDL-control, incubated with copper (10 μM) for 0, 1, 2 and 8 h. (B) LDL-treated, incubated with copper (10 μM) in the presence of 320 μg/ml of VAPE. Experiments were repeated three times and the gel shown is typical of the results obtained.

Fig. 4. Fluorescence anisotropy [r] of HDL (A) and LDL (B) incubated (treated) or not (control) in the presence of VAPE (320 μg/ml) overnight. Results represent mean ± S.E.M. of three independent experiments. *P < 0.05, **P < 0.01.
Human and animal studies strongly support the hypothesis that oxidative modification of LDL plays a crucial role in the pathogenesis of atherosclerosis. High levels of oxidized LDL are found in various acute coronary syndromes, indicating that oxidized LDL might be a marker for atherosclerosis [28].

Epidemiological studies have shown that consumption of food and beverages rich in phenols can reduce the risk of heart disease by slowing the progression of atherosclerosis [26].

Our results show that VAPE-PE is very efficient in the protection of LDL against lipid peroxidation as demonstrated by the decrease in conjugated diene and MDA formation. This protective effect can occur via several mechanisms, either via: (A) the scavenging of peroxy radicals, which break the peroxidation chain reaction, (B) chelating free Cu\(^{2+}\) to form redox-inactive complexes and thus reducing metal-catalyzed oxidation of LDL, (C) inhibiting the binding of copper ions to apolipoproteins and subsequently preventing the modification of amino acid-apo-B protein residue. These hypotheses are also supported by the extended lag phase, the reduction in the oxidation rate of the propagation phase and the maximal accumulation of oxidation products, in the presence of VAOE-PE.

VAOE-PE decreases the rate of disappearance of \(\alpha-\) and \(\gamma-\) tocopherol and preserves endogenous Vitamin E in LDL. The same mechanism of action was shown for ascorbate, a watersoluble vitamin, which preserves tocopherols and \(\beta\)-carotene in LDL [33].

The antioxidant activity of polyphenolics is principally defined by the presence of orthohydroxy substituents, which stabilize radicals and chelate metals. The antioxidant effect of phenolic acids and their esters depends on the number of hydroxyl groups in the molecule. Argan oil, with comparison to olive oil, contains a higher quantity of ferulic acid (3470±13 versus 51±2 µg/kg of oil, respectively) [16]. This acid is more effective than ascorbic acid and other phenolic acid such as \(p\)-coumaric acid, since the electron-donating methoxy group allows increased stabilization of the resulting aryloxyl radical through electron delocalization after hydroxyl donation by the hydroxyl group [34]. This showed that the direct inhibition of trans-conjugated diene hydroperoxide isomer formation is related to the H-donating ability of the phenol [35]. Argan oil, but not olive or sunflower oil, also contains another important phenolic acid: syringic acid (68±4 µg/kg). This antioxidant compound protects against LDL-oxidation [36]. Fito et al. [37] demonstrated that phenolic compounds from olive oil could associate with LDL and inhibit LDL oxidation. This association can protect LDL in removing excess cholesterol from macrophage cells.

Argan oil contains a high amount of Vitamin E but also a non-negligible proportion of phenolic compounds. We have previously demonstrated that chronic ingestion of crude argan oil not only reduces plasma cholesterol and LDL levels in rats fed with hypercholesterolemic diet [18], but also improve endothelial function and prevents high blood pressure [19]. These effects are principally related to the richness of argan oil in oleic and linoleic acids and \(\alpha-\) and \(\gamma-\)tocopherol. However, the antioxidant activity of phenolic compounds remains unknown. Our experiments were designed to examine the antioxidant activity of VAPE-PE in inhibiting LDL oxidation and their effect on HDL in removing excess cholesterol from macrophage cells.
after LDL incubation with ferulic acid, 14% of the ferulic acid is incorporated into LDL, and 86% stays in the LDL-aqueous phase, making a strong association between phenolic compounds and the protein-fraction of LDL.

LDL oxidation is characterized by alterations in the structural and biological properties of lipids and apoprotein B (apoB). This alteration starts with fragmentation of the protein, which contains sensitive amino-acids residues, and is followed by cross-linking of reactive aldehydes (MDA) and oxysterols (end products of the lipid peroxidation) [39]. We explored the inhibitory effect of VAO-PE on the copper-induced alteration of apoB. Our results show that VAO-PE abolishes the Cu^{2+}-induced electrophoretic shift in LDL, indicating that there was a protection from oxidative modification of LDL. As mentioned above, VAO-PE inhibits the maximal production of oxidation product in a dose dependent manner and therefore protects the apoprotein moiety against alteration. Moreover, VAO-PE can also act by either blocking the copper-binding site or by binding to another (allosteric) site and thus reducing the binding of copper. Alternatively, VAO-PE may interfere with the reoxidation activity of copper ions on the surface of the lipoprotein and exert similar effects to those obtained by blocking the copper-binding site.

Oxygen free radicals induce lipid peroxidation and disrupt important structural and protective functions associated with bio-membranes. This oxidation is implicated in various in vivo pathological events [40]. Lipoprotein can be protected against oxidation by reducing fatty acid oxidation and stabilizing the lipid bilayer. Our results show that incubation of LDL and HDL with VAO-PE increases the fluidity in the LDL and HDL phospholipidic bilayer. This result suggests that the protective effect of VAO-PE towards LDL peroxidation could be due in part to a membrane-stabilizing activity. Indeed, a recent study suggests that another factor contributing to the antioxidant effectiveness of certain phenolic compounds is their degree of incorporation, uniformity of distribution and orientation in the membrane bilayer [40].

Few studies have investigated the effect of antioxidants on the biological activities of HDL and especially on reverse cholesterol transport. In this context, the physicochemical characteristics of HDL determine their capacity to remove free cholesterol from cells. Indeed, cholesterol efflux capacity increases with the fluidity of HDL, which in turn depends on the length and saturation of fatty acids in the HDL composition [41]. Previous studies have suggested that when HDL are oxidized, a process that leads to a loss of polysaturated fatty acids, their capacity to remove free cholesterol from cells is decreased, due in part to a reduction in HDL fluidity [42]. This fluidity is linked to lipoprotein structure, which is maintained by the phopholipid and fatty acid content.

Taking these mechanisms together, we postulate that VAO-PE enhances cholesterol efflux from THP-1 by protecting HDL from oxidation and increasing their fluidity and thus the binding of HDL to cell membranes. VAO-PE can also interact directly with THP-1 membrane and/or penetrate the membrane, thus inducing modification of the lipid bilayer and lipid-protein interactions.

In conclusion, VAO-PE protects LDL from oxidation by a direct or indirect antioxidant activity. VAO-PE increases cholesterol efflux by increasing HDL lipid-bilayer fluidity. However, further studies are needed to clarify the exact action of VAO-PE on lipoprotein oxidation and reverse cholesterol transport. These results support the use of argan oil as a dietary supplement.

Acknowledgements

This work was supported by grants from the Canadian Institutes of Health research (CIHR) and from the research center on aging. This support is gratefully acknowledged. Abdelouahed Khalil is junior 2 fellow of FRSQ.

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