The hypolipidemic natural product Commiphora mukul and its component guggulsterone inhibit oxidative modification of LDL

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Abstract

There is accumulating evidence that LDL oxidation is essential for atherogenesis, and that antioxidants that prevent this oxidation may either slow down or prevent atherogenesis. In the present study, we found that Commiphora mukul and its cholesterol-lowering component, guggulsterone, effectively inhibited LDL oxidation mediated by either catalytic copper ions, free radicals generated with the azo compound 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH), soybean lipoxygenase enzymatically, or mouse peritoneal macrophages. This inhibition was assessed by the decrease in the following parameters describing LDL oxidation: conjugated dienes, relative electrophoretic mobility (REM), thiobarbituric acid reactive substances, lipid hydroperoxides, oxidation-specific immune epitopes as detected with a monoclonal antibody against oxidized LDL, and the accumulation of LDL derived cholesterol esters in mouse peritoneal macrophages. We concluded that C. mukul and its lipid-lowering component, guggulsterone, significantly inhibit LDL oxidation. The combination of antioxidant and lipid-lowering properties of C. mukul and guggulsterone makes them especially beneficial against atherogenesis.

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Keywords: Commiphora mukul; Guggulsterone; Low-density lipoprotein; Antioxidant; Macrophages

1. Introduction

Atherosclerosis is the principal contributor to the pathogenesis of myocardial and cerebral infarction, gangrene and loss of function in the extremities. The prevailing explanation for early atherogenesis has been the "response-to-injury" hypothesis [1], which proposes that there are various forms of stimulants to the endothelium and smooth muscle cells of the artery wall, and atherosclerotic lesions result from an excessive, inflammatory-fibroproliferative response to these stimulants. Later, a "response-to-retention" hypothesis of early atherogenesis proposes that only the retention of lipoproteins in the arterial wall is both necessary and sufficient for atherogenesis, while endothelial injury and other factors are either not necessary or not sufficient [2].

LDL has been found to accumulate in atherosclerotic lesions and is the major source of the cholesterol accumulating in human foam cells. A growing body of evidence indicates that, before it can induce either the formation of foam cells or atherogenesis, LDL must be modified, most likely oxidized [3]. As a result of discoveries that LDL is oxidized in vivo, and that oxidized LDL is actively involved in atherogenesis [4], antioxidants have been used to inhibit LDL oxidation in vitro and to significantly arrest atherogenesis in rabbits, hamsters, mice and non-human primates in vivo [5].

The extract from the bark of Guggul (Commiphora mukul) tree found native in India has been used as a valued herb in Ayurvedic medicine for over 2500 years. It has been used [6]: to reduce a possible cause of sluggishness and inflamed joints by removing deposits of waste and toxic material from the body, including mucus and mineral deposits from the joints; as an antiseptic, antirheumatic, and anti-inflammatory drug; to induce weight loss; as a uterine stimulant; as an emmenagogue, expectorant, astringent, aperient, demulcent, and as a gargle in tooth disease, chronic tonsillitis, and ulcerated throat. It also increases thermogenesis by stimulating...
the thyroid. In the past decades, the C. mukul has been found to lower total and LDL cholesterol and triglycerides, and to inhibit platelet aggregation [7]. It is available in the United States as a dietary supplement for cholesterol-lowering, weight loss and arthritis treatment. Recently, it was found that the basis for the lipid-lowering property of C. mukul is that its active component, guggulsterone, is an antagonist ligand for the nuclear receptor farnesoid X receptor (FXR) [8,9] and bile acid receptor (BAR) [10].

In the present study, we hypothesized that C. mukul contains antioxidants, which might account for its wide usefulness in many diseases because reactive oxygen species are responsible for mediating a great variety of diseases. Specifically, we tested whether C. mukul and guggulsterone are able to increase the resistance of LDL to oxidative modifications and consequently retard macrophage foam cell formation. We found that both C. mukul and guggulsterone significantly inhibited LDL oxidation and thus could retard macrophage foam cell formation.

2. Methods

2.1. Materials

C. mukul extract (referred to as C. mukul in this study), which contained 14.2% guggulsterone E isomer and 4.50% guggulsterone Z isomer, was purchased from Laila Impex (Vijayawada, India), and guggulsterone Z and guggulsterone E and Z mixture were gifts from Frank Jaksch Jr. at Chromadex (Santa Ana, CA). Bovine serum albumin (BSA) and soybean lipoxygenase (type IB) were purchased from Sigma. Thioglycollate medium, Dulbecco’s modified Eagle medium (DMEM), Ham’s F-10 medium, and fetal calf serum (FCS) were purchased from Gibco BRL. Tissue culture plates were purchased from Costar, 2′-azobis-(2-amidinopropane)dihydrochloride (AAPH) from Polysciences Inc., Europium salt from Lambda Probes, CHOD-iodide color reagent from Merck, and Sephadex G-25 from Pharmacia Biotech.

2.2. Lipoprotein preparation

Plasma was donated by normolipidemic humans whose serum lipoprotein(a) levels were lower than 1 mg/100 ml. LDL and lipoprotein-deficient serum (LPDS) were isolated from this plasma by differential ultracentrifugation at density ranges between 1.020–1.050 and >1.235 g/ml, respectively [11]. Protein concentration of LDL was measured by the Lowry method using BSA as standard. LDL concentration is referred to as its protein content.

2.3. Cell cultures

 Resident peritoneal macrophages (MPM) from BALB/c mice were harvested 3 days after i.p. injection of 2 ml of 3% thioglycollate medium. Primary cultures were prepared in 24-well plates at a density of 1 × 106 cells/well, each well containing DMEM, 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cells were maintained in a humidified incubator with 5% CO2 at 37°C. Three hours after the cells were plated, non-adherent cells were washed out with 10 ml/0.1 PBS pH 7.4. The adherent cells were cultured overnight in the same medium as described above. Before they were used, the cells were washed three times with PBS. Cell viability (assessed by trypan blue exclusion) was greater than 98%.

2.4. Modification of LDL

Prior to copper oxidation, LDL was dialyzed at 4°C against 10 mmol/l phosphate buffer (pH 7.4) that had been degassed and saturated with nitrogen gas. Stock solution was made by dissolving either C. mukul or guggulsterone powder in DMSO at 1 mg/ml. In our preliminary study, we found that at the same concentration (25 μg/ml), the mixture of Z and E guggulsterone (containing equal amount of Z and E isomers) were equally effective as Z-guggulsterone alone in inhibiting LDL oxidation as judged with conjugated diene assay, suggesting Z and E isomers were equally effective. Z-guggulsterone was used for the studies described here. Three types of LDL mixtures were then prepared: one with 1–50 μg/ml of C. mukul, one with 1–50 μg/ml (3.2–160 μM) of Z-guggulsterone, and one (controls) with neither. Each of these mixtures was incubated for 30 min at 37°C.

2.4.1. Oxidizing LDL with Cu2+ ions

Samples of each LDL mixture were incubated with CuCl2·2H2O (20 μmol/l) at 37°C for 4 h, periodically removed, and the reactions were terminated by adding EDTA to a final concentration of 0.27 mmol/l. The samples were saturated with nitrogen and stored at 4°C until the measurements for oxidative modification (see below).

2.4.2. Oxidizing LDL with free radicals

Samples of each LDL mixture were incubated with AAPH 10 mmol/l) at 37°C for up to 24 h. AAPH is a water-soluble azo compound that thermally decomposes to produce per-oxyl radicals at a constant rate in the water phase. Sample aliquots of the reaction were periodically removed, and the oxidative modification of LDL was immediately estimated.

2.4.3. Oxidizing LDL with lipoxygenase

Samples of each LDL mixture were incubated with soybean lipoxygenase (11,060 U/ml) either with or without 60 μg/ml of linoleic acid in PBS at 37°C for up to 24 h. Sample aliquots of the reaction were periodically removed, and the oxidative modification of LDL was immediately estimated.

2.4.4. Oxidizing LDL with MPM

Samples of each LDL mixture were added to Ham’s F-10 medium, and incubated with macrophages in 24-well plates
(0.6 ml/well) for up to 24 h. Sample aliquots of the media were periodically removed, and the oxidative modification of LDL was measured. It was impossible to measure LPO concentrations because of interference from culture medium. Control LDL samples in PBS without the oxidation reagents or in the medium in the absence of cells were incubated for the same period of time as were those with the reagents or in the presence of cells.

2.5. Determination of oxidative modification of LDL

LDL oxidation was estimated with the following five indices: (1) conjugated dienes: \(\text{Cu}^{2+} (10 \mu\text{mol/l})\) or AAPH (2 mmol/l) were added into LDL (50 \(\mu\text{g/mL}\)) with different concentrations of either \(C. mukul\) or \(Z\)-guggulsterone, and the formation of conjugated dienes was continuously monitored at 234 nm in a temperature-controlled (37 °C) spectrophotometer (Hitachi U-2000) [12]; (2) lipid hydroperoxide (LPO) concentrations, measured at 365 nm with a spectrophotometric assay using a CHOD-iodide color reagent [13]; (3) thiobarbituric acid reactive substances (TBARS) concentrations, expressed as malondialdehyde (MDA) equivalents (nmol/mg LDL protein) [13]; (4) relative electrophoretic mobility (REM) compared with native LDL, estimated on 1% agarose gels at pH 8.05 with the Lipidophor system (Immuno AG) [13]; (5) oxidation-specific epitopes, measured with a solid-phase sandwich fluorescence assay [13]. Briefly, 200 \(\mu\text{l}\) of LDL (10 \(\mu\text{g/ml}\)) in 10 mmol/l PBS, pH 7.4 containing 1 g/l EDTA was incubated in each well of microtitration plates at 4 °C overnight. The monoclonal antibody OB/04 (IgG) against oxidized LDL [14] and rabbit IgG against apoB (Behring AG) were used as detecting antibodies. After washing the plates three times, Eu \(^{3+}\)-labeled rabbit anti-mouse IgG (for OB/04) or Eu \(^{3+}\)-labeled sheep anti-rabbit IgG (for anti-apoB) were used as reporting antibodies by incubation for 1 h at 25 °C. After washing and addition of the enhancement solution (Wallac Oy), fluorescence was measured.

2.6. Measurement of cellular cholesterol

MPM were cultivated in 24-well plates (Costar) in DMEM containing 10% FCS. Total and free cholesterol content of the cells were measured with a fluorometric assay [11].

3. Results

3.1. \(C. mukul\) and \(Z\)-guggulsterone inhibited the formation of conjugated dienes in LDL upon \(\text{Cu}^{2+}\)- and AAPH-mediated oxidation in a dose-dependent manner

\(C. mukul\) and \(Z\)-guggulsterone inhibited \(\text{Cu}^{2+}\)- and AAPH-mediated LDL oxidation in a dose-dependent manner (Fig. 1). In the absence of \(C. mukul\) and \(Z\)-guggulsterone, the lag time for \(\text{Cu}^{2+}\)-mediated LDL oxidation was 22 min (calculated with the method of Esterbauer et al. [15]). In the presence of 1, 2, 10, and 25 \(\mu\text{g/ml}\) of \(C. mukul\), it increased to 29, 34, 64, and 130 min, respectively (Fig. 1A); in the presence of 10, 25, and 35 \(\mu\text{g/ml}\) of \(Z\)-guggulsterone, it increased to 50, 59, and 79 min, respectively (Fig. 1B). In the absence of \(C. mukul\) and \(Z\)-guggulsterone, the lag time for AAPH-mediated LDL oxidation was 25 min. In the presence of 2 and 20 \(\mu\text{g/ml}\) of \(C. mukul\), it increased to
Table 1

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<td>1.46 ± 0.12</td>
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<td>3.21 ± 0.11</td>
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<td>1.46 ± 0.08**</td>
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<td>3.33 ± 0.12</td>
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<td>2.18 ± 0.03**</td>
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<td>1.04 ± 0.08*</td>
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<td>1.38 ± 0.17**</td>
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LDL (300 μg/ml) was incubated either with 20 μg/ml of C. mukul (+ C. mukul) or without it (−C. mukul, controls) at 37 °C for 1 h. After that, LDL was oxidized by either Cu2+ (20 μmol/l), AAPH (10 mmol/l), soybean lipoxygenase (LOX; 11,060 U/ml) in PBS, or by mouse peritoneal macrophages (MPM) in Ham’s F-10 medium in 24-well plates. Samples were taken out after different time intervals and measured for the electrophoretic mobility relative to non-oxidized LDL on 1% agarose gel. Data are expressed as means ± S.D. from three separate experiments. Compared with those of controls (−C. mukul) (calculated with two-tailed Student’s t-test).

* P < 0.05.
** P < 0.01.

45 and 112 min, respectively (Fig. 1C); in the presence of 25 and 35 μg/ml of Z-guggulsterone, it increased to 39 and 105 min, respectively (Fig. 1D). Thus at the same dosage, C. mukul is more effective than Z-guggulsterone in inhibiting LDL oxidation.

3.2. C. mukul decreased the electrophoretic mobility of LDL upon oxidation

The relative electrophoretic mobility of LDL incubated with C. mukul and either copper, AAPH, soybean lipoxygenase, or mouse peritoneal macrophages increased continually but significantly less (P < 0.05) over 24 h than did that of LDL comparably incubated but without C. mukul (Table 1).

3.3. C. mukul inhibited TBARS formation in LDL upon oxidation

TBARS in LDL continuously increased in the first 24 h when LDL was subjected to oxidation mediated by either copper, AAPH, soybean lipoxygenase, or mouse peritoneal macrophages. C. mukul significantly (P < 0.05) reduced the amount of TBARS formed in this time period (Table 2).

3.4. C. mukul decreased LPO formation in a dose-dependent manner during LDL oxidation

When LDL was oxidized by copper, LPO concentrations initially increased. As the labile LPO intermediates decomposed to a variety of products, including many aldehydes, LPO concentrations decreased (Fig. 2A). Formation of LPO was either slowed down (copper oxidation group, Fig. 2A) or decreased (AAPH oxidation group, Fig. 2B) when LDL was pre-incubated with C. mukul, compared with when LDL was comparably oxidized but without the...
C. mukul pre-incubation, and the effect was *C. mukul* dose-dependent.

3.5. *C. mukul* inhibited the formation of oxidation-specific immune epitopes in LDL in a dose-dependent manner during oxidation

As LDL is oxidized, the ratio of oxidation-specific epitopes to native apolipoprotein increases and can be quantified with a sandwich immuno-fluorescence assay. This ratio was considerably less when LDL was oxidized by either 

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<td>−<em>C. mukul</em></td>
<td>0.95 ± 0.12</td>
<td>48.32 ± 6.30</td>
<td>52.75 ± 3.39</td>
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<td>+<em>C. mukul</em></td>
<td>1.02 ± 0.25</td>
<td>15.17 ± 2.17**</td>
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<td>−<em>C. mukul</em></td>
<td>0.88 ± 0.19</td>
<td>6.17 ± 1.25</td>
<td>9.66 ± 0.87</td>
<td>11.14 ± 0.99</td>
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<td>+<em>C. mukul</em></td>
<td>0.91 ± 0.15</td>
<td>3.62 ± 0.68**</td>
<td>8.34 ± 1.01</td>
<td>8.81 ± 0.82**</td>
<td>9.99 ± 0.73**</td>
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<td>−<em>C. mukul</em></td>
<td>0.94 ± 0.06</td>
<td>0.81 ± 0.12</td>
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<td>13.01 ± 1.04</td>
<td>20.54 ± 2.47</td>
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<td>5.53 ± 1.44*</td>
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MPM

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<td>2.72 ± 0.73**</td>
<td>3.07 ± 0.88**</td>
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LDL (500 μg/ml) was incubated either with 20 μg/ml of *C. mukul* (+*C. mukul*) or without it (−*C. mukul*, controls) at 37°C for 1 h. After that, LDL was oxidized by either Cu²⁺ (20 μM), AAPH (10 mM), soybean lipoygenase (LOX; 11,060 U/ml) in PBS, or by mouse peritoneal macrophages (MPM) in Ham’s F-10 medium in 24-well plates. Samples were taken out after different time intervals and measured for the TBARS (nmol MDA/mg LDL protein). Data are expressed as means ± S.D. of three separate experiments. Compared with those of controls (−*C. mukul*) (calculated with two-tailed Student’s *t*-test).

* P < 0.05.  ** P < 0.01.

C. mukul significantly inhibited the production of TBARS due to LDL oxidation mediated by either copper, AAPH, soybean lipoygenase (LOX), or mouse peritoneal macrophages (MPM).

Table 2

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<td>2.72 ± 0.73**</td>
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On the other hand, macrophages cultured with LDL and *C. mukul* accumulated significantly less esterified cholesterol than those with LDL but without *C. mukul*, the amount accumulated being inversely proportional to the dose of *C. mukul* in the medium. The amount of esterified cholesterol in cells cultured with EDTA (which inhibits LDL oxidation) was similar to the basal cellular amount, implying that mouse peritoneal macrophages accumulate esterified cholesterol because they oxidize LDL.

4. Discussion

One major risk factor for atherosclerosis is the elevated level of LDL, the principal carrier of plasma cholesterol in humans. Paradoxically, native LDL fails to exert potentially atherogenic effects. Oxidation of LDL, however, renders the lipoprotein atherogenic [3]. Four major lines of evidence have demonstrated that oxidation of LDL does indeed occur in vivo [4]. (1) oxidized LDL has been found in atherosclerotic lesions from both rabbits and humans, and a minor fraction of LDL in circulating plasma showed signs of oxidative modification; (2) antibodies against oxidized...
LDL react with materials from atherosclerotic lesions, but not with those from normal arteries; (3) autoantibodies against oxidized LDL have been found in the plasma and atherosclerotic lesions of humans and rabbits, and T lymphocytes from human atherosclerotic plaques recognize oxidized LDL; and (4) antioxidants slow the progression of atherosclerotic lesions of humans and rabbits, and T lymphocytes from human atherosclerotic plaques recognize oxidized LDL; and (4) antioxidants slow the progression of atherosclerosis. It has been shown to have adverse biological effects, many of which could mediate atherogenesis [4] : it induces foam cell formation; it is mitogenic for macrophages and smooth muscle cells; it inhibits the motility of tissue macrophages; it is cytotoxic; it is an antioxidant and effective for lipid disorders [6] . The recent finding that its lipid-lowering component, guggulsterone, is an antagonist for FXR [8,9] and bile acid receptor [10] has triggered great interest in this ancient drug. Guggulsterone induces bile salt export pump (BSEP) [9,20] and orphan nuclear receptor small heterodimer partner (SHP) [9], inhibits human CYP7A1 gene by activation of pregane X receptor (PPAR) [20]. While these may explain why C. mukul is effective for lipid disorders, it is still a mystery why C. mukul is effective for such a variety of other diseases.

Unfortunately, the finding of oxidized LDL in vivo may have adverse biological effects, many of which can mediate atherogenesis [4]: it induces foam cell formation; it is a chemotactant for monocytes and inhibits the motility of tissue macrophages; it is cytotoxic; it is an antioxidant and effective for lipid disorders; and (4) antioxidants slow the progression of atherosclerosis. It has been shown to have adverse biological effects, many of which could mediate atherogenesis [4]: it induces foam cell formation; it is mitogenic for macrophages and smooth muscle cells; it inhibits the motility of tissue macrophages; it is cytotoxic; it is an immunogenic; and it induces a wide array of immune reactions.

Not surprisingly, the finding of oxidized LDL in vivo and the various adverse biological effects of oxidized LDL in atherogenesis has stimulated the studies on antioxidants against LDL oxidation, hoping to either slow down or prevent atherosclerosis. Although most of the large-scale, double-blind, placebo-controlled trials have shown that vitamin E and β-carotene (either alone or combined with other antioxidant vitamins) do not reduce the risk of fatal or nonfatal myocardial infarction [17–19], the oxidative modification hypothesis of atherosclerosis cannot be refuted because not knowing the mechanism by which LDL is oxidized in vivo, the optimal antioxidant regimens cannot be determined [17,18].

The medicinal properties of C. mukul are not new. Its has been used for more than 2500 years to treat a wide variety of diseases, including lipid disorders [6]. The recent finding that its lipid-lowering component, guggulsterone, is an antagonist for FXR [8,9] and bile acid receptor [10] has triggered great interest in this ancient drug. Guggulsterone induces bile salt export pump (BSEP) [9,20] and orphan nuclear receptor small heterodimer partner (SHP) [9], inhibits human CYP7A1 gene by activation of pregane X receptor (PPAR) [20]. While these may explain why C. mukul is effective for lipid disorders, it is still a mystery why C. mukul is effective for such a variety of other diseases.

Coincidentally, reactive oxygen species are responsible for mediating a great variety of diseases, including atherosclerosis, hypertension, rheumatoid arthritis, inflammatory bowel disease, adult respiratory distress syndrome, cancers, Parkinson’s disease, and Alzheimer’s disease [21], which might suggest that C. mukul is an antioxidant and thus effective for so many diseases.

Therefore, in this study, we explored whether C. mukul can act as an antioxidant. We specifically studied whether C. mukul and its lipid-lowering guggulsterone component
could inhibit LDL oxidation. Although it is not clear how LDL is oxidized in vivo, the following may be involved: metal ions (copper and/or iron) [22–25], lipooxygenase [26–28], myeloperoxidase (MPO) [29,30], superoxide radicals, reactive nitrogen species, and reduced thiols [31–33]. To represent these putative mechanisms, we subjected LDL to oxidation by copper, lipooxygenase, free radicals directly generated by AAPH, and macrophages (which might employ one or more of those mechanisms). We were able to demonstrate that C. mukul significantly inhibited LDL oxidation in all these model oxidation systems. Furthermore, we found that C. mukul decreased the accumulation of LDL-derived cholesterol esters in macrophages in the presence of C. mukul.

The mechanism by which C. mukul inhibits oxidation is unknown. Because it could inhibit both AAPH- and 15-lipoxygenase-mediated LDL oxidation in the absence of transition metal ions, C. mukul may act as a chain-breaking free radical scavenger. However, it may also act as an antioxidant partly by chelating metal ions.

The chemical composition of C. mukul is very complex and has not been well defined. It may contain sugars (starch, fructose), amino acids, camphorine, cembrene, cembroid, resin, oils, and several steroids or sterones. Only some steroid components have been purified, including Z- and E guggulsterones which have been shown to be responsible for the cholesterol- and lipid-lowering effects of C. mukul [6]. Although we showed that guggulsterone alone inhibited LDL oxidation, C. mukul surely contains other antioxidants because it is more effective in preventing LDL oxidation than guggulsterone. In future studies, it is important to analyze and purify the active ingredients in C. mukul and explore its active antioxidant ingredients.

In summary, we found that C. mukul and guggulsterone are effective antioxidants against LDL oxidation. The combination of antioxidant and lipid-lowering properties of C. mukul and guggulsterone makes them especially beneficial against atherogenesis. Therefore, in the current and future clinical trials on C. mukul, guggulsterone and guggulsterone analogs, oxidation indices should be included in the study design. In addition, our study suggests that the antioxidant property of C. mukul might partly explain its effectiveness in such a wide variety of diseases.

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