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Clerodendron glandulosum.Coleb extract prevents *in vitro* human LDL oxidation and oxidized LDL induced apoptosis in human monocyte derived macrophages

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ABSTRACT

This study reports the protective role of *Clerodendron glandulosum* (CG) extract against *in vitro* LDL oxidation and Ox-LDL induced macrophage apoptosis using various experimental models. Effect of CG extract on Cu^{2+} mediated LDL oxidation kinetics and formation of various intermediary products and its ability to prevent human monocyte derived macrophage mediated LDL oxidation have been investigated. Ox-LDL induced macrophage apoptosis was evaluated by nuclear condensation, cell cycle analysis, and annexin V-FITC/PI staining in presence or absence of CG extract. Results recorded in the present study clearly suggest the protective role of CG extract against LDL oxidation and Ox-LDL induced macrophage oxidative stress, mitochondrial dysfunction, and apoptosis. This is the first report on the protective role of CG extract on two key events of atherosclerosis portending its possible therapeutic use as an anti-atherogenic herbal medicine.

1. Introduction

Oxidation of LDL is crucial in plaque formation and onset of atherosclerosis (Steinberg, 1997), a cardiovascular disorder posing as the leading cause of death in developed as well as developing countries (Stachura and Pierzynowski, 2009). A number of effects like inhibition of motility of resident macrophages, induction of monocyte differentiation into macrophages, promotion of its uptake by macrophages through scavenger receptors, and causation of subsequent cytotoxicity have all been reported for Ox-LDL (Young and McEneny, 2001). Asmis and Begley (2003) have reported cytotoxicity of Ox-LDL in the form of apoptosis of monocyte derived macrophages. Apparently, macrophage apoptosis plays a key role in development of atherosclerotic lesion as several studies have demonstrated occurrence of macrophage apoptosis in human atheroma (Isner et al., 1995; Kolodgie et al., 1999). Natural antioxidants and lipid lowering interventions such as food supplements, spices, and herbs find extensive application in prevention of atherosclerosis because of their ability to prevent *in vitro* LDL oxidation (Chang et al., 2006; Chu et al., 2009) and plaque formation (Ho et al., 2010).

Clerodendron glandulosum.Coleb (CG; Fam: Verbenaceae) is an herb endemic to North-Eastern region of India and commonly known as "*Kuthab laba*". Apatani and Nyishi tribes of North-East India use leaves of CG as a therapeutant against hypertension (Kala, 2005; Deb et al., 2009) and, a cross sections of people across Manipur traditionally consume a decoction of CG leaves for treating various ailments like diabetes, obesity and hypertension (Jadeja et al., 2009a). Recent studies from our laboratory have demonstrated many favorable effects of CG aqueous extract against fructose induced insulin resistance and hypertension (Jadeja et al., 2010a), high fat diet induced hyperlipidemia in rats (Jadeja et al., 2010b) and hepatic steatosis (Jadeja et al., 2010c) and obesity (Unpublished observations) in C57BL/6J mice, besides its free radical scavenging activity (Jadeja et al., 2009b). Moreover, safety evaluation of CG aqueous extract in Swiss albino mice has revealed its LD_{50} indices to be higher than 3000 mg/kg (Jadeja et al., 2011).

These reports have established the therapeutic property of CG extract in combating metabolic disorders and cardiovascular diseases and, the present study in continuation investigates the efficacy of CG aqueous extract as an anti-atherosclerotic agent using *in vitro* experimental models.

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidized LDL; HMDMs, human monocyte derived macrophages; PBS, phosphate buffer saline; MDA, malonaldehyde; LHP, lipid hydroperoxide; CD, conjugated diene; PC, protein carbonyl; ApoB, apolipoprotein B100; nLDL, native LDL; EDTA, ethylene diamine tetra acetic acid; BHT, butylated hydroxytoluene; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulphoxide; DCF-DA, dichlorodihydrofluorescein diacetate; REM, relative electrophoretic mobility; PUFA, poly unsaturated fatty acid.

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2. Materials and methods

2.1. Chemicals

Na_2Co_3, folin ciocalteau, $AlCl_3$, potassium acetate, sodium chloride, $Cuso_4$, EDTA, TBA, TCA, BHT, SDS, DNPH, bromophenol blue and coomassie brilliant blue were purchased from Sisco Research Laboratories, Mumbai, India. HCl, H_2SO_4, heptanes, ethyl acetate, glacial acetic acid and glycerol were purchased from Suvidhanath Laboratories, Baroda, India. Barbituric acid and Na-barbiturate were purchased from National Chemicals, Baroda, India. PBS, PMA, RPMI-1640, fetal bovine serum, DMSO, MTT, Ham's F12 media and antimicrobial–antimycotic solution were purchased from Himedia Laboratories, Mumbai, India. DCF-DA, rhodamine-123, DAPI, Rnase A, and annexin V/FITC-propidium iodide staining kit were purchased from Sigma–Aldrich, USA. Heparin (Hep-25) was purchased from Gland Pharma Ltd., Hyderabad, India.

2.2. Plant material and preparation of extract

CG leaves collected from Imphal district India in the month of June were shade dried. Dr. Hemchand Singh, Taxonomist, Department of Botany, D.M. College of Science Manipur, Imphal, identified the plant and a sample (voucher specimen No. 405) was deposited at the herbarium of the Department of Botany. For preparation of extract, CG leaves were shade dried, crushed, and ground in an electric grinder to obtain a fine powder. The powder (100 g) boiled in 1 l of distilled water at 100 °C for 3 h was filtered using a sterilized muslin cloth. The filtrate collected in petri plates was concentrated by heating at 100 °C to form a semisolid paste. This paste was kept overnight at 0 °C and a freeze dried extract was obtained. The net yield obtained at the final step of extraction was 28% W/W.

2.3. Estimation of polyphenols and flavonoids in CG

Total polyphenolic content was determined as per the method of Chandler and Dodds (1993). Whereas, total flavonoid content of CG extract was determined by the aluminum chloride colorimetric method of Chang et al. (2002).

2.4. Isolation of human LDL

Venous blood was collected from fasting individuals, kept at room temperature for 45 min, and centrifuged at 3000 rpm for 10 min at 4 °C, to obtain serum. LDL was isolated by heparin–citrate buffer (0.064 M tri sodium citrate at pH 5.05 containing 50,000 IU/l heparin) precipitation method as described earlier by Ahotupa et al. (1998). To 0.1 ml serum 1 ml of the heparin–citrate buffer was added, mixed with a vortex mixer and allowed to stand for 10 min at room temperature. The insoluble lipoproteins were sedimented by centrifugation at 3000 rpm for 10 min (at 20 °C) and the resultant pellet was suspended in 1 ml PBS (0.1 M, pH 7.4, containing 0.9% NaCl). Protein concentration of the LDL was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.4.1. LDL oxidation kinetics

Hundred micro liter of LDL (100-μg protein) diluted to 0.9 ml with PBS was incubated with or without 100 μl of CG extract (20–200 μg/ml) at 37 °C for 30 min. At the end of incubation, oxidation was initiated by adding 10 μl of freshly prepared 0.167 mM $CuSO_4$. The oxidation kinetics were determined by continuously monitoring the change in absorbance every 10 min at 37 °C for 3 h at 234 nm for the production of CD in a UV/VIS Perkin–Elmer spectrophotometer as described by Esterbauer et al. (1989).

Lag time (min) was determined from the intercept of lines drawn through the linear portions of the lag phase and propagation phase. The rate of oxidation (expressed as nmol/min) was determined from the slope of the propagation phase. Maximum concentration of CD formed (CD_{max} = expressed as nmol/mg protein) was calculated from the difference in absorbance at zero time and at diene peak. The concentration of CD in the samples was calculated by using a molar extinction coefficient of $2.95 \times 10^4\ M^{-1}\ cm^{-1}$ (Esterbauer et al., 1989).

2.4.2. Measurement of MDA, LHP and PC content

Three sets of tubes were prepared for LDL oxidation. Copper mediated LDL oxidation was carried out in presence or absence of CG extract (10–200 μg/ml) for 24 h as mentioned above. Later, 10 μl of 10 mM EDTA was added in each tube to stop oxidation and each one was processed for measurement of MDA, LHP and PC.

For MDA measurement, 100 μl aliquot was mixed with 1 ml TBA reagent (0.37% TBA, 15% TCA in 0.25 N HCl) and placed in water bath at 100 °C for 30 min, cooled to room temperature and centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm with UV/VIS Perkin–Elmer spectrophotometer and, MDA was calculated using a molar absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Buege and Aust, 1978).

For LHP estimation, 100-μl aliquot was mixed with 0.9 ml of Fox reagent (250-μM ammonium sulfate, 100-μM xylenol orange, 25 mM H_2SO_4 and 4 mM BHT in 90% (v/v) HPLC-grade methanol) and incubated at 37 °C for 30 min. The color developed was read at 560 nm and LHP content was determined using the molar absorption coefficient of $4.3 \times 10^4\ M^{-1}\ cm^{-1}$ (Nourooz-Zadeh et al., 1996).

For PC estimation, 0.1 ml aliquot was mixed with 0.2 ml of DNPH (in 2 M HCl). After incubation at room temperature for 1 h, 0.6 ml denaturing buffer (0.15 M sodium phosphate buffer containing 3% SDS) was added and mixed thoroughly. Then ethanol and heptane (1.8 ml of each) were added, mixed for 1 min, and centrifuged to precipitate protein. The protein was washed three times with 1.5 ml ethylacetate/ethanol (1/1, v/v) and dissolved in 1 ml denaturing buffer and read at 360 nm in a spectrophotometer. The carbonyl content was calculated from the absorbance (360 nm) using an absorption coefficient ε of 22,000 $M^{-1}\ cm^{-1}$ (Reznick and Packer, 1994).

2.4.3. Relative electrophoretic mobility assay

Copper mediated LDL oxidation was carried out in presence or absence of CG extract (10–200 μg/ml) for 24 h as mentioned above. The electrophoretic mobility of native or oxidized LDL (with or without CG extract) was detected by agarose gel electrophoresis (Reid and Mitchinson, 1993). Samples were loaded on 0.6% agarose gel and electrophoresed (100 V) in 50 mM barbituric acid (pH 8.6) for 30 min. After electrophoresis, the gels were fixed in a solution containing 60% methanol, 30% water, and 10% glacial acetic acid for 30 min. The gels were then dried at 50 °C for 40 min in a hot air oven and stained with 0.6% Sudan black B for 60 min and photographed. The result was expressed in terms of distance moved from origin.

2.4.4. ApoB fragmentation assay

Copper mediated LDL oxidation was carried out in presence or absence of CG extract (10–200 μg/ml) for 24 h as mentioned above. The LDL samples were denatured with 3% SDS, 10% glycerol, and 5% bromophenol at 95 °C for 10 min. Later, LDL samples were subjected to SDS–PAGE (8%) electrophoresis at 100 V for 60 min and the gels were stained with coomassie brilliant blue (Lee et al., 2002).

2.5. Preparation of oxidized LDL and human monocyte derived macrophage (HMDM)

Hundred micro liter of LDL (100 μg protein) diluted to 900 μl with PBS was incubated for 24 h at 37 °C subsequent to initiation of oxidation by 10 μl of freshly prepared 0.167 mM $CuSO_4$. Analysis of MDA and CD was carried out in the LDL samples. Samples with MDA 50 ± 5 nmol/mg LDL protein and CD 80 ± 8 nmol/mg LDL protein were used for further studies.

THP-1, human monocyte cell line, was purchased from National Centre of Cell Sciences, Pune (India). Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution in a humidified incubator with 5% CO_2. THP-1 monocyte cells were differentiated into macrophages by the addition of 50 nM phorbol 12-myristate 13-acetate (PMA) for 48 h (Park et al., 2007). After differentiation to macrophages, the PMA-containing medium was replaced with serum-free medium and the cells were cultured for another 24 h before treatment.

2.5.1. Cell mediated LDL oxidation

HMDMs (1×10^5/24 mm well) underwent incubation in 1 ml of Ham's F-12 medium (without phenol red) containing nLDL (100 μg/ml) with or without CG extract at 37 °C for 24 h. Cell free control well was used for all conditions. At the end of incubation, oxidation was arrested by chilling the medium and adding 200 μM EDTA and 40 μM BHT. About 100 μl of each supernatant was used for the assay of MDA and CD as described earlier (Duell et al., 1998).

2.5.2. Ox-LDL induced foam cell formation

HMDMs pre-treated with CG extract (200 μg/ml) for 30 min were incubated in presence of 100 μg/ml of Ox-LDL for 24 h. Later, medium was decanted and cells fixed in 4% paraformaldehyde for 15 min. The cells were then washed twice with PBS, and stained in 1% Oil red O solution for 30 min. At the end of staining, excess Oil red O was removed and 1 ml of glycerin added. Photographs were taken on Leica DMIL inverted microscope using canon power shot S 70 digital camera.

2.5.3. Ox-LDL induced cytotoxicity of HMDMs

HMDMs pre-treated with CG extract (10–200 μg/ml) for 30 min were incubated in presence of 100 μg/ml of Ox-LDL for 24 h. Further incubation of the cells was carried out in a culture medium containing 0.5 mg/ml MTT for 4 h. Later, 150 μl of DMSO was added to all the wells and, were incubated for 30 min at room temperature with constant shaking. Absorbance was read at 540 nm using ELX800 Universal Microplate Reader (Bio-Tek instruments, Inc., Winooski, VT) and subsequently % cell viability was calculated.