

The Journal of Phytopharmacology

(Pharmacognosy and phytomedicine Research)

Research Article

ISSN 2320-480X
JPHYTO 2018; 7(1): 79-83
January- February
Received: 30-12-2017
Accepted: 16-02-2018
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Cuminum cyminum methanolic extract prevents oxidative modification of low density lipoproteins: Preliminary evidence on its anti-atherosclerotic potential

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ABSTRACT

The significance of oxidative modification of LDL in the pathogenesis of atherosclerosis and the lack of efficient treatment intervention has led researchers to develop an effective therapy based on natural anti-oxidants. The present study provides preliminary evidence in support of the anti-atherosclerotic potential of methanolic extract of *Cuminum cyminum* L. (CC). We found that CC inhibited Cu²⁺-mediated LDL oxidation as demonstrated by the *ex vivo* LDL oxidation kinetic study, the LDL oxidation products (malondialdehyde, lipid hydroperoxide and protein carbonyl), and ApoB fragmentation assay. It can be concluded that, CC efficiently alleviates experimentally induced oxidative changes and modifications of LDL. Since oxidative changes in LDL are prerequisite to onset of atherogenic changes, this study provides preliminary evidence on anti-atherosclerotic potential of CC.

Keywords: *Cuminum cyminum*, LDL oxidation, ApoB fragmentation.

INTRODUCTION

Atherosclerosis is a multifactorial disease primarily characterized by deposition of fatty substances in the arterial wall leading to the narrowing of its lumen. The elevated levels of plasma low density lipoproteins (LDL) caused by dysregulated lipid metabolism is one of the key factors contributing to complex etiology of atherosclerosis [1]. Furthermore, the impairment of anti-oxidant defense mechanisms along with the generation of reactive oxygen species (ROS) causes oxidative modification of LDL forming oxidized LDL (Ox-LDL), which is taken up by macrophages to form foam cells initiating inflammatory events. As the disease progresses, the foam cells undergo apoptotic death, further contributing to the inflammatory milieu of the subendothelial space by recruiting more macrophages to the site [2]. Thus, it is evident that oxidative stress acts as the initiator of atherosclerosis and hence, it appears that antioxidants that inhibit LDL oxidation may be effective as therapy against atherosclerosis.

Active antioxidant constituents of have been in focus of research for their therapeutic potentials including anti-atherosclerotic properties. Till date, our lab had reported anti-atherosclerotic potential of *Clerodendron glandulosum* [3], *Sida rhomboides*. Roxb [4], *Coriandrum sativum* L. [5] and *Murraya koenigii* [6]. *Cuminum cyminum* (fam. Apiaceae) is an annual herb whose seeds are widely used in Indian culinary and are also reported for their medicinal properties. Cumin seed extract (CC) have been reported to be effective against skin rashes, kidney and gallbladder stones, nausea toothaches, hiccoughs, dyspepsia, diarrhea, epilepsy, jaundice, flatulence and indigestion. Furthermore, essential oil of CC has been reported to have diuretic, carminative, emmenagogic, antibacterial, antifungal, antioxidant, anticancer, antidiabetic and antispasmodic properties [7, 8]. Besides being anti-oxidant, CC has been reported for its lipid lowering activity [9]. This study investigates efficacy of CC in preventing oxidative modifications of LDL by assaying LDL oxidation products and ApoB fragmentation. The aim was to gather preliminary evidence on therapeutic potential of CC in an experimentally simulated condition wherein lipotoxicity is at the epicenter of pathophysiology.

MATERIALS AND METHODS

Chemicals and reagents

Sodium carbonate, folin's reagent, acetic anhydride, trisodium citrate, ammonium sulphate, xylenol orange, potassium acetate, sodium chloride, copper sulphate, ethylene di-amine tetrachloro acetic acid

(EDTA), thiobarbituric acid (TBA), trichloroacetic acid (TCA), butylated hydroxyanisole (BHT), sodium dodecyl sulphate (SDS), di-nitrophenyl hydrazine (DNPH), heptane, ethyl acetate, chloroform, iron (III) chloride, phosphomolybdic acid, acrylamide, bisacrylamide, ammonium persulphate, tetramethylethylenediamine (TEMED), glycerol, bromophenol blue and coomassie brilliant blue R250 were purchased from Sisco Research Laboratories (Mumbai, India). Hydrochloric acid (HCl), sulphuric acid (H₂SO₄), and glacial acetic acid were purchased from Suvadhan Laboratories, (Vadodara, India). Tris base and glycine were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Preparation of plant extract

CC seeds were collected from Spencer's mall, Vadodara, Gujarat, India, identified and authenticated by Dr. Vinay Raole, Department of Botany and voucher specimen (accession number 379) was submitted to departmental herbarium (BARO), The M. S. University of Baroda, Vadodara, Gujarat, India. 100 g dried seeds were powdered, defatted (in 70% petroleum ether overnight) and extracted with 80% methanol in a soxhlet apparatus to obtain a flavonoid rich extract. The resultant extract was concentrated in a rotary evaporator, followed by hydrolysis in a waterbath (at 60°C) for 24 h that yielded two phases. The organic phase (flavonoid rich) was used for further investigation [10]. The final yield was 8.3 g (w/w).

Qualitative phytochemical analysis

CC extract was subjected to qualitative phytochemical analysis for detection of major chemical groups such as phenolic acids (phosphomolybdic acid test), alkaloids (Dragendorff's test), flavonoids (Shinoda test), glycosides (Keller-kilani test), terpenoids and steroids (Liebermann-Burchardt test), saponins (Frothing test) and proteins and amino acids (copper sulphate test) as described previously [11, 12].

Isolation of LDL

LDL was isolated using heparin-citrate buffer precipitation method [13]. Briefly, blood was collected under the supervision of a pathologist from normocholesterolemic healthy adult volunteers (n=6) under fasting condition after taking prior consent and as per the standard guidelines at Blue cross pathology lab (IMA-BMWMC No. 1093). Serum was obtained after centrifugation (3000 rpm for 10 min at 4°C) and 0.1 ml was mixed with 1 ml heparin-citrate buffer (64 mM trisodium citrate at pH 5.05 containing 50,000 IU/ml heparin), vortexed and allowed to stand for 10 min at room temperature. Later, the mixture was centrifuged at 3,000 rpm for 10 min at 20 °C and the resultant pellet was re-suspended in 0.1 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.4, containing 0.9 % NaCl). The protein concentration of the obtained LDL was estimated [14] using bovine serum albumin as a standard.

LDL oxidation kinetics

100 µg/ml of LDL was incubated with or without 0.1 ml of various concentrations of CC extract (10–300 µg/ml) at 37 °C for 30 min and subsequently, 10 µl freshly prepared 0.167 mM Copper sulfate solution. Absorbance was recorded by continuous monitoring (at 10 min interval for 180 min at 234 nm using UV-Vis Perkin Elmer spectrophotometer) to record LDL oxidation kinetics. The lag time was determined from the intercepts of lines drawn through the linear

portions of the lag phase and propagation phase, whereas the rate of oxidation was determined from the slope of the propagation phase. The concentration of conjugate diene (CD) in the samples was calculated by using a molar extinction coefficient of $2.95 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. Maximum concentration of CD formed was calculated from the difference in the concentration of CD at time 0 min and at absorption maxima [15].

Determination of LDL oxidation products

The LDL was oxidized in presence or absence of CC extract (10–300 µg/ml) for 24 h as mentioned above. Later, 10 µl of 10 mM ethylene diamine tetra acetic acid (EDTA) was added to stop oxidation reaction and the samples (in triplicates) were subjected to quantification of LDL oxidation products viz. malondialdehyde (MDA), lipid hydroperoxide (LHP) and protein carbonyl (PC) as follows:

For MDA estimation, 0.1 ml of oxidized LDL (Ox-LDL) was mixed with 1 ml TBA reagent (0.37 % TBA, 15 % TCA in 0.25N HCl) and incubated (at 100 °C for 30 min), cooled and centrifuged at 3000 rpm for 10 min. The absorbance was read at 532 nm (in UV-Vis Perkin Elmer spectrophotometer) and the amount of MDA was calculated using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [16].

For LHP estimation, 0.1 ml of Ox-LDL was mixed with 0.9 ml of Fox reagent (0.25 mM ammonium sulphate, 0.1 mM xylenol orange, 25 mM H₂SO₄, and 4 mM BHT in 90 % (v/v) HPLC-grade methanol) and incubated at 37 °C for 30 min. The absorbance was recorded at 560 nm and LHP content was determined using the molar extinction coefficient of $4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ [17].

For estimation of PC, 0.1 ml of aliquot was mixed with 0.2 ml of DNPH (in 2 M HCl). After incubation at room temperature for 60 min, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer containing 3 % SDS) was added and mixed thoroughly. This was followed by addition of ethanol and heptane (1:1; 1.8 ml each) and the contents were centrifuged at 3,500 rpm to precipitate protein. The protein was washed thrice with 1 ml ethyl acetate/ethanol (1:1, v/v), dissolved in 1 ml of denaturing buffer, and read at 360 nm using UV-Vis Perkin Elmer spectrophotometer. The PC content was calculated using molar extinction absorption coefficient of $22.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [18].

Apolipoprotein B100 (ApoB) fragmentation assay

ApoB fragmentation assay was carried out to check the oxidative fragmentation of ApoB of LDL. Briefly, LDL was oxidized in presence or absence of CC extract (10–300 µg/ml) for 24 h and the reaction was stopped using 10 mM EDTA as mentioned above. LDL obtained after centrifugation was denatured with sample buffer (3 % SDS, 10 % glycerol, and 5 % bromophenol) at 95 °C for 5 min and cooled to room temperature. All the LDL samples were subjected to electrophoretic separation using 8 % SDS-PAGE. The gels were stained (2 % Coomassie brilliant blue R250 solution) and de-stained (20 % glacial acetic acid and 10 % methanol in water) followed by its imaging using Bio-Rad gel documentation system [19].

Statistical analysis

All the experiments were performed in triplicates. Statistical evaluation of the data was done by one-way ANOVA followed by Bonferroni's multiple comparison tests. The results were expressed as

mean ± S.E.M and the statistical analysis was carried out using Graph Pad Prism version 3.0.

RESULTS

Phytochemical constitution of CC extract

The results of the preliminary phytochemical analysis are shown in Table 1.

Table 1: Qualitative assessment of phytochemical constituents of CC extract.

Phytochemical	Test results
Alkaloids	Absent
Phenolic compounds	Highly present
Phytosteroids	Poorly available
Proteins and amino acids	Absent
Saponins	Absent
Glycosides	Present
Flavonoids	Present

Cu²⁺-induced LDL oxidation

We observed a dose-dependent decrease in the rate of Cu²⁺-mediated oxidation of LDL in presence of CC (Fig. 1). Further, a significant increase in the lag period and decrease in the CD_{max} values of LDL oxidation was observed in samples containing CC (Fig. 1). Moreover, increase in LDL oxidation products (MDA, LHP and PC) was recorded on addition of CuSO₄ but CC extract supplementation recorded a dose dependent decrement in the same (Fig. 2). Absence of ApoB band suggested its fragmentation in Ox-LDL group whereas, its presence was recorded in the sample containing CC (Fig. 3).

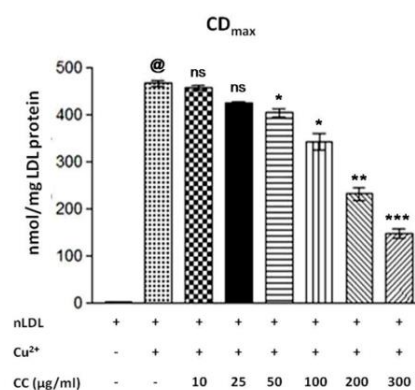
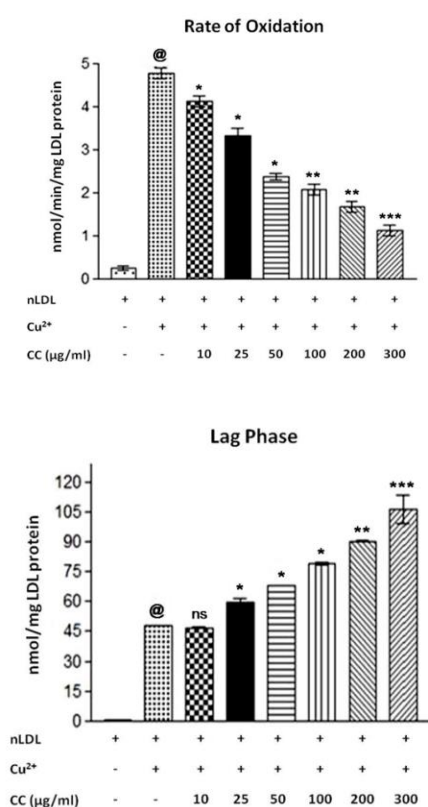


Figure 1: Effect of CC on LDL oxidation kinetics (lag time period, rate of oxidation and maximum conjugated diene formation; CD_{max}) in Cu²⁺ induced LDL oxidation. Data were expressed as mean ± S.E.M. for n=3. @p<0.001 compared to nLDL, ns non-significant, *p<0.05, **p<0.01, ***p<0.001 compared to Ox-LDL.

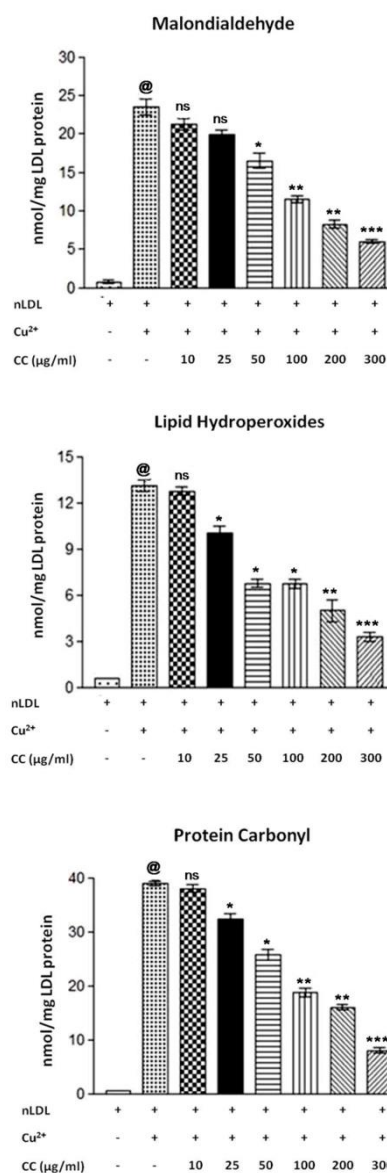


Figure 2: Effect of CC on LDL oxidation end products (malondialdehyde, lipid hydroperoxides and protein carbonyls) in Cu²⁺ induced LDL oxidation. Data were expressed as mean ± S.E.M. for n=3. @p<0.001 compared to nLDL, *p<0.05, **p<0.01, ***p<0.001 compared to Ox-LDL.

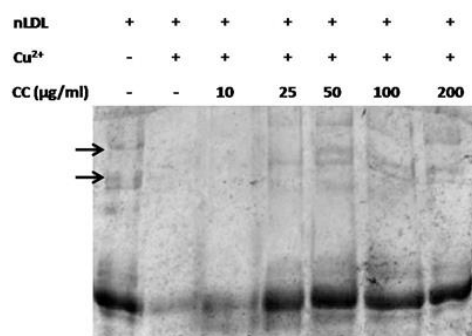


Figure 3: Effect of CC on ApoB fragmentation in Cu²⁺ mediated oxidized LDL. Arrow indicates ApoB protein band in SDS-PAGE.

DISCUSSION

Progression of atherosclerosis in vivo is preceded by elevated levels of circulating cholesterol coupled with low grade inflammation [20] or environmental pollutant [21]. The sequence of events often overlap and make the study challenging. Oxidative modification of LDL is one such event that has been extensively studied and prevention of the same by a therapeutant is believed to retard the atherosclerotic cascade. Covalent modification lipids lead to harmful intermediate products that are toxic to endothelial cells, monocytes, neutrophils, etc. and restrict macrophage mobility [1, 22]. Oxidation of lipid moieties also produce lipid peroxides that cause rearrangement of fatty acid double bonds and production of conjugated dienes (CD). Also, the resultant aldehydes and ketones produced by fatty acid fermentation cause ApoB fragmentation [1].

Copper mediated LDL oxidation is a widely used protocol to obtain prima facie evidence on potential of test compound in preventing LDL oxidation. In our study, an extended lag phase recorded in presence of CC indicates at its ability in delaying Cu²⁺ mediated LDL oxidation and minimizing the resultant CD. Further, a dose dependent decrement in LDL oxidation products (MDA, LHP, PC) in presence of CC is in agreement with the previous observation. Overall, CC appears to play a protective role as evidenced by significantly less formed LDL oxidation products.

Oxidative deletion of peptide bonds in an LDL molecule leads to its alteration and ApoB fragmentation. This process also form covalent adducts and increases the net negative charge of the molecule. Since the same is recognized by the scavenger receptors of the macrophages that follows foam cell formation [23], reduction/prevention of ApoB fragmentation is an important credential in a test compound. In our study, the electrophoretic profile showed disappearance of the ApoB band in Ox-LDL sample however, a dose-dependent recovery of the ApoB band was seen in CC supplemented groups. These results are attributable to high content of flavonoids and phenolic compounds in CC reported herein. Hence, CC is thought to be instrumental in preventing oxidative modification of LDL molecules due to free radical scavenging activity of the phenolic compounds and flavonoids present in its extract.

CONCLUSION

It can be concluded from this study that CC is a promising prospect as an anti-atherosclerotic agent and needs a detailed study to establish the same.

Acknowledgements

Authors are thankful to Gujarat State Biotechnology Mission (GSBTM), Gandhinagar, Gujarat, India for providing financial support for the study in the form of Financial Assistance Program (FAP)-2010 (GSBTM/MD/PROJECTS/SSA/457/2010-2011) to RD. University Grant Commission (UGC), New Delhi, India is also acknowledged for providing Rajiv Gandhi National Fellowship (RGNF) to KS.

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HOW TO CITE THIS ARTICLE

Devkar R, Lagu K, Thadani J, Shirsath K. *Cuminum cyminum* methanolic extract prevents oxidative modification of low density lipoproteins: Preliminary evidence on its anti-atherosclerotic potential. *J Phytopharmacol* 2018; 7(1):79-83.