Curcuma oil ameliorates hyperlipidaemia and associated deleterious effects in golden Syrian hamsters

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Abstract

Essential oil components from turmeric (Curcuma longa L.) are documented for neuroprotective, anti-cancer, anti-thrombotic and antioxidant effects. The present study aimed to investigate the disease-modifying potential of curcuma oil (C. oil), a lipophilic component from C. longa L., in hyperlipidaemic hamsters. Male golden Syrian hamsters were fed a chow or high-cholesterol (HC) and fat-rich diet with or without C. oil (30, 100 and 300 mg/kg) for 28 d. In HC diet-fed hamsters, C. oil significantly reduced plasma total cholesterol, LDL-cholesterol and TAG, and increased HDL-cholesterol when compared with the HC group. Similar group comparisons showed that C. oil treatment reduced hepatic cholesterol and oxidative stress, and improved liver function. Hyperlipidaemia-induced platelet activation, vascular dysfunction and repressed eNOS mRNA expression were restored by the C. oil treatment. Furthermore, aortic cholesterol accumulation and CD68 expression were also reduced in the C. oil-treated group. The effect of C. oil at 300 mg/kg was comparable with the standard drug ezetimibe. Delving into the probable anti-hyperlipidaemic mechanism at the transcript level, the C. oil-treated groups fed the chow and HC diets were compared with the chow diet-fed group. The C. oil treatment significantly increased the hepatic expression of PPARα, LXRXα, CYP7A1, ABCA1, ABCG5, ABCG8 and LPL accompanied by reduced SREBP-2 and HMGR expression. C. oil also enhanced ABCA1, ABCG5 and ABCG8 expression and suppressed NPC1L1 expression in the jejunum. In the present study, C. oil demonstrated an anti-hyperlipidaemic effect and reduced lipid-induced oxidative stress, platelet activation and vascular dysfunction. The anti-hyperlipidaemic effect exhibited by C. oil seems to be mediated by the modulation of PPARα, LXRXα and associated genes involved in lipid metabolism and transport.

Key words: Curcuma oil: Hamsters: Hyperlipidaemia: PPARα: Liver X receptor α

Turmeric (Curcuma longa L.) is one of the most widely used ancient herbs, which is traditionally used in several Asian countries for several inflammatory, infectious, fungal and viral ailments. Various preparations derived from turmeric display potential therapeutic effects against cancer, pain, stomach upset, ulcer, dysentery and wounds(1). Previous work from our institute demonstrated the isolation and characterisation of curcuma oil (C. oil) components. The major constituents of C. oil are ar-d-turmerone and α/β-turmerone(2–4), while other minor constituents are curcumene, zingiberene, germacrone, curcumerone, zedoarone, sedoarondiol, isozedoarondiol, curcumene and curcurnone(2,3,5). The neuroprotective effect of C. oil has been shown in a rat model of cerebral ischaemia–reperfusion injury(6–8), which is mediated by the inhibition of NO synthase expression, NO content and oxidative stress(6,7). Moreover, C. oil and its components have been shown to exhibit several favourable effects on proliferation(9–11), inflammation(12), oxidation(12) and platelet activation(14). Keeping in mind the therapeutic array of C. oil and its components, we tested its effect on hyperlipidaemia and associated deleterious changes. Cholesterol homeostasis in the body is mostly regulated by the nuclear receptor superfamily of transcription

Abbreviations: AA, arachidonic acid; ABC, ATP binding cassette; C. oil, curcuma oil; CYP7A1, cholesterol 7α-hydroxylase; eNOS, endothelial NO synthase; FC, free cholesterol; HC, high cholesterol; HDL-C, HDL-cholesterol; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL-C, LDL-cholesterol; LPL, lipoprotein lipase; LXRXα, liver X receptor; MDA, malondialdehyde; NPC1L1, Niemann–Pick C1-like 1; SREBP-2, sterol regulatory element-binding protein 2; TC, total cholesterol.

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factors such as PPAR and liver X receptors (LXR)(13). The activation of PPARα by natural or synthetic ligands regulates hepatic lipid metabolism, reduces intestinal cholesterol absorption(14) and increases faecal cholesterol excretion(14), and thereby decreases plasma and tissue lipid accumulation(15,15). LXR positively regulate several hepatic and intestinal genes involved in cholesterol metabolism and excretion from the body(13). LXR activation has also been shown to promote ‘macrophage-to-faeces’ reverse cholesterol transport in hyperlipidaemic hamsters(16). We and others have demonstrated golden Syrian hamsters as a valuable preclinical model of dietary-induced hyperlipidaemia, and that it is well suited for the screening of anti-hyperlipidaemic agents(17–19). In addition, hamsters also bear a resemblance to human plasma lipid distribution, synthesis and excretion(17,18). In the present study, we evaluated the anti-hyperlipidaemic effect of C. oil on hyperlipidaemia and associated complications in golden Syrian hamsters.

Materials and methods

Materials

The Amplex Red Cholesterol Assay kit was obtained from Invitrogen, Molecular Probes. The RevertAid™ H Minus first-strand cDNA synthesis kit and SYBR green maxima were obtained from Thermo Fischer Scientific, Fermentas, Inc. Acetylcholine chloride, phenylephrine hydrochloride and ADP were purchased from Sigma-Aldrich. Equine tendon fibrillar collagen type I and arachidonic acid (AA) were procured from Chrono-Log Corporation. Anti-phosphotyrosine clones, PY20 and 4 G10, were obtained from Santa Cruz Biotechnology and Millipore, respectively.

Animal diet and treatment

The preparation and quality assessment of C. oil were performed as described earlier(3,18). Golden Syrian hamsters (110–115 g) obtained from the National Laboratory Animal Centre at the Council of Scientific and Industrial Research-Central Drug Research Institute, Lucknow, India, were used and received humane care in compliance with the Guidelines for the Care and Use of Laboratory Animals. The hamsters were kept in polypropylene cages at 24 ± 0.5°C and a 12 h day–12 h night cycle, and were given ad libitum access to water and food. The experimental protocols were approved by the Institutional Animal Ethics Committee, which follow the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals and conform to the international norms of the Indian National Science Academy. Initially, hamsters were allowed to acclimatise for 7 d with free access to water and a chow diet containing protein, carbohydrate, fat, vitamins, minerals and fibre as described earlier(19). After acclimatisation, the animals were randomly divided into four groups; the first two groups were kept on a chow diet alone or a chow diet along with C. oil (300 mg/kg per d) up to 28 d. Hamsters of the other two groups were fed with a high-cholesterol (HC) diet (chow diet supplemented with 1% cholesterol and 15% saturated fat (coconut oil)). After 7 d of the HC diet treatment, plasma total cholesterol (TC) was estimated and the animals exhibiting almost similar plasma cholesterol concentrations were regrouped for another 28 d as follows: a HC diet-fed alone; a HC diet along with C. oil (30, 100 and 300 mg/kg per d) or ezetimibe (1 mg/kg per d). C. oil or ezetimibe was administered orally (0.5 ml/animal per d) in 0.25% carboxymethyl cellulose sodium suspension, and carboxymethyl cellulose sodium alone was taken as the vehicle control. At least twelve animals were analysed in each group.

Plasma and serum biochemistry

Blood samples from the overnight-fasted animals were collected and centrifuged at 5000 rpm for 10 min to obtain plasma. TC, LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C) and TAG along with alanine aminotransferase, and aspartate aminotransferase were estimated in the plasma using the Beckman Coulter, Synchron CX9 Pro, Biochemistry Analyzer (Beckman Coulter, Inc.) and commercial kits. Plasma malondialdehyde (MDA), an indicator of oxidative stress, was measured spectrophotometrically as described earlier(20). Briefly, 250 µl of plasma mixed with 300 µl of 30% TCA, 150 µl of 5 M-HCl and 300 µl of 2% (w/v) 2-thiobarbituric acid were heated for 8 min at 90°C. After centrifugation at 12,000 rpm for 10 min, a pink-coloured supernatant was collected and colour intensity was measured spectrophotometrically at 532 nm(20).

Appraisal of vascular function

Endothelial function (vasoconstriction and vasodilatation) was monitored in the control and treated animals as described previously(19,20). In brief, transverse 4 mm-wide rings of the thoracic aorta were cut and mounted in 10 ml organ baths containing Krebs solution. After equilibration, the aortic rings were exposed to KCl Krebs buffer (80 mM) in order to assess the maximum tissue contractility. The presence of a functional endothelium was then verified by the occurrence of significant relaxation to acetylcholine (3 mM–3 mM) in phenylephrine (1 µM)-pre-contracted rings. Cumulative concentration-dependent contraction responses to phenylephrine were also assessed. Finally, tissue contractility and viability were assessed by exposing the rings to KCl Krebs buffer (80 mM) in all groups(21).

Aortic and liver cholesterol estimation

After collecting the blood, the animals were perfused with cold PBS containing 5 mM-EDTA. Liver and the whole aorta were removed, cleaned and weighed, and lipid was extracted with hexane–isopropanol (3:2)(22). The extracted lipids were dried and resuspended in reagent-grade ethanol containing NP40 (9:1). Tissue TC and free cholesterol (FC) were measured using the cholesterol assay kit according to the manufacturer’s protocol. In brief, 50 µl of samples were incubated with 50 µl of working reagent from the cholesterol assay kit for 30 min n the dark. After incubation, the plate was read.
by means of a fluorescence plate reader (BMG LABTECH GmbH) at an excitation wavelength of 540 nm and 590 nm as the emission wavelength. Cholesteryl ester was derived after subtracting FC from TC.

Whole blood aggregation and static platelet adhesion
Whole blood aggregation was performed in the citrated blood using a dual-channel aggregometer (Chrono-Log Corporation) as described previously(19). Aggregation was induced by ADP (10 μM), collagen (2·5 μg/ml) and AA (0·25 mM) followed by measuring impedance over a time interval of 6 min. Static platelet adhesion was measured as the number of platelets adhered on a collagen- or fibrinogen-coated surface as described earlier(19). The adhered platelets were measured spectrophotometrically using p-nitrophenyl phosphate(19).

Immunoblotting
Phosphotyrosine blotting was performed in platelets from the chow diet- and HC diet-fed hamsters with or without C. oil (300 mg/kg) as described previously(4). In brief, platelet activation was triggered in washed platelets by collagen (5 μg/ml) followed by stopping the reaction with sample buffer (2% SDS, 0·062 M-Tris–HCl, 0·01% bromphenol blue, 10% glycerol and 20% β-mercaptoethanol, pH 6·8) containing 2 mM-phenylmethyl sulfonyl fluoride, 10 mM-sodium fluoride and 1 mM-sodium orthovanadate. The samples were run on SDS–PAGE (8%) and transferred onto a nitrocellulose membrane (Bio-Rad), blocked with Tris-buffered saline with 5% bovine serum albumin for 1 h, and then probed with primary antibodies for 2 h: anti-p-Tyr (57 Kim et al., 1999), b-actin (diluted 1:10 000 in TBST).

Semi-quantitative and real-time quantitative RT-PCR
Total RNA was extracted from the liver, small intestine (jejunum) and thoracic aorta of the different groups of experimental hamsters using the TRIZOL isolation procedure as described previously(19). Complementary DNA was synthesised using the RevertAid™ H Minus first-strand complementary DNA synthesis kit (Thermo Fischer Scientific, Fermentas, Inc.) according to the manufacturer’s protocol. To explore the possible underlying mechanism of C. oil-induced plasma and tissue lipid lowering, the mRNA expression of various genes was quantified using specific primers (Table 1). To assess the effect on cholesterol synthesis, metabolism and transport, the hepatic mRNA expression of PPARα, lipoprotein lipase (LPL), sterol regulatory element-binding protein 2 (SREBP-2), 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGC R), LDL receptor (LDLR) and cholesterol 7α-hydroxylase (CYP7A1) were monitored. For cholesterol absorption and efflux, LXRα, ATP binding cassette (ABC) transporters such as ABCA1, ABCG5 and ABCG8, and Niemann-Pick C1-like 1 (NPC1L1) were monitored(25).

The effect of C. oil on endothelial NO synthase (eNOS) and macrophage content was determined by evaluating the mRNA expression of eNOS (conventional end-point RT-PCR(19) and CD68, respectively, in the thoracic aorta. The real-time RT-PCR was carried out using the LightCycler® 480II Real-Time PCR system (Roche Applied Science) along with SYBR green maxima reagents. The amplification conditions used in the present study consisted of an initial pre-incubation at 94 or 95°C for 10 min followed by the amplification of the target DNA for forty-five cycles (95°C for 10 s and 57–60°C (as applicable) for 10 s). Melting curve analysis was performed immediately after amplification using the manufacturer’s protocol(24).

Table 1. Primer sequences used for mRNA expression

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα</td>
<td>GGGCAATGGCATCCAAAATAA</td>
<td>CTTGGCAGATCTGTGAGC</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>LPL</td>
<td>GATTCACCTTCTTGGCGACTGA</td>
<td>GCCACTGTGCGTACAGA</td>
<td>59</td>
<td>Mukherjee et al. (52)</td>
</tr>
<tr>
<td>SREBP-2</td>
<td>GCAGGAGGTCTCTCAGTAGAA</td>
<td>TGTTGCTTCACTGATCAGCC</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>HMGC R</td>
<td>GAGCTACATTTCTCTGCTGACC</td>
<td>TTCATAGCGCGAGCTCAC</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>LDLR</td>
<td>GCAGTGGTCTGGCTGACGAC</td>
<td>GCCATGCAAGGGTCCA</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>LXRX</td>
<td>TCAGCCTCTTCTCCTGACGACC</td>
<td>TCATTAGCAGCTGGACA</td>
<td>59</td>
<td>Li et al. (56)</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>CACTCTGAGCTCTTGTGACC</td>
<td>GGCTGCTGAGTAGGCA</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATAGAGGGGCTCGACCATCTGTGCAG</td>
<td>GGTCTAGAGCATTTGTGCTGTT</td>
<td>60</td>
<td>Lecker et al. (57)</td>
</tr>
<tr>
<td>ABCG5</td>
<td>TGATTGGGCTGAATTATTTTGGG</td>
<td>GTGTCGGCGAGTAGA</td>
<td>60</td>
<td>Lecher et al. (57)</td>
</tr>
<tr>
<td>NPC1L1</td>
<td>CCTGACCTTTATAGAATCCACACAGA</td>
<td>GCCGCGATATGCGTTCAT</td>
<td>60</td>
<td>Lecher et al. (57)</td>
</tr>
<tr>
<td>eNOS</td>
<td>GGCTGCTCACCTACCTGGCGACC</td>
<td>GGATCCTGGAGAAGGCGTGGAGG</td>
<td>61</td>
<td>Marinho et al. (51)</td>
</tr>
<tr>
<td>CD68</td>
<td>CAGAGGATGATCCTCCTGCGG</td>
<td>CTTGATGACGTGTCCTGCT</td>
<td>57</td>
<td>Kim et al. (52)</td>
</tr>
<tr>
<td>b-Actin</td>
<td>TGCTGCCCTGATGA</td>
<td>AGGGAGCGAGCTGCCC</td>
<td>58</td>
<td>Lecher et al. (57)</td>
</tr>
</tbody>
</table>

LPL, lipoprotein lipase; SREBP-2, sterol regulatory element-binding protein 2; HMGC R, 3-hydroxy-3-methyl-glutaryl-CoA reductase; LDLR, LDL receptor; LXRα, liver X receptor α; CYP7A1, cholesterol 7α-hydroxylase; ABCA1, ATP binding cassette A1; ABCG5, ATP binding cassette G5; ABCG8, ATP binding cassette G8; NPC1L1, Niemann–Pick C1-like 1; eNOS, endothelial NO synthase; CD68, cluster of differentiation 68.

* Conventional RT-PCR.

Statistical analysis
All experimental results were reproduced in at least eight to twelve animals for each parameter. Results are expressed as means with their standard errors. The statistical significance...
of difference between the different groups was determined by one-way ANOVA followed by Bonferroni’s post hoc test using GraphPad Prism 5 software (GraphPad, Inc). The significance level for Bonferroni’s multiple comparison test was set to 0·05 for three or more groups, and P≤0·05 was considered as statistically significant.

Results

Curcuma oil reduced diet-induced plasma and hepatic cholesterol levels

Continuous HC diet feeding for 35 d significantly increased the circulating levels of TC, LDL-C, HDL-C and TAG (P<0·001; Fig. 1(a)). The anti-hyperlipidaemic effect of C. oil was tested on plasma lipids at the three different doses of 30, 100 and 300 mg/kg. The lower dose of C. oil (30 mg/kg) was ineffective in regulating the plasma and tissue lipid levels. C. oil (100 mg/kg) effectively reduced TC and LDL-C (P<0·05); however, plasma TAG and HDL-C remained unchanged at this dose (Fig. 1(a)). The higher dose of C. oil (300 mg/kg) used in the present study exhibited a significant reduction in plasma TC, LDL-C (P<0·001) and TAG (P<0·05) and increased HDL-C (P<0·05; Fig. 1(a)). The administration of ezetimibe (1 mg/kg) in HC diet-fed hamsters showed a significant reduction in plasma TC, LDL-C (P<0·001) and TAG (P<0·05; Fig. 1(a)).

Cholesterol lipid-rich liver is the hallmark of hyperlipidaemia; therefore, the effect of C. oil was evaluated on hepatic lipid accumulation. Hyperlipidaemic hamsters showed a remarkable increase in liver FC and cholesteryl esters compared with the chow diet-fed group (P<0·001). C. oil at both 100 and 300 mg/kg doses significantly reduced hepatic TC, FC and cholesteryl esters (P<0·05 and P<0·001, respectively; Fig. 1(b)). Similarly, in the ezetimibe-treated group, there was a significant decline in hepatic lipid accumulation (P<0·001; Fig. 1(b)) compared with the HC diet-fed group.

Table 2. Curcuma oil (C. oil) attenuates oxidative stress and improves liver function

<table>
<thead>
<tr>
<th>Group</th>
<th>Liver/body weight ratio</th>
<th>MDA (µM)</th>
<th>Liver function test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean SE</td>
<td>Mean SE</td>
<td>Mean SE</td>
</tr>
<tr>
<td>Chow</td>
<td>0·03 0·002</td>
<td>0·34 0·05</td>
<td>58·9 4·1</td>
</tr>
<tr>
<td>HC</td>
<td>0·06 0·007</td>
<td>0·53 0·06</td>
<td>217·6 31·4</td>
</tr>
<tr>
<td>HC + C. oil-30 mg/kg</td>
<td>0·06 0·008</td>
<td>0·56 0·10</td>
<td>191·6 28·1</td>
</tr>
<tr>
<td>HC + C. oil-100 mg/kg</td>
<td>0·04 0·005</td>
<td>0·40 0·03</td>
<td>127·1 22·3</td>
</tr>
<tr>
<td>HC + C. oil-300 mg/kg</td>
<td>0·03 0·002</td>
<td>0·36 0·04</td>
<td>66·2 9·8</td>
</tr>
</tbody>
</table>

MDA, malondialdehyde; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HC, high cholesterol.

Mean values were significantly different from the chow diet-fed animals (one-way ANOVA): * P<0·05, ** P<0·01, † P<0·001.

Mean values were significantly different from the HC diet-fed animals (one-way ANOVA): † P<0·05, †† P<0·01, ††† P<0·001.

† mmol oxaloacetate released/min per litre of serum.

§ mmol pyruvate released/min per litre of serum.

Curcuma oil attenuates hyperlipidaemia-induced oxidative stress and liver dysfunction

Plasma MDA is widely considered to be a reliable biomarker for oxidative stress. Therefore, to assess the effect of C. oil on oxidative stress, we estimated plasma MDA levels. Consumption of the HC diet resulted in enhanced plasma MDA (P<0·05), indicating general oxidative stress under hyperlipidaemia. This increase in plasma MDA was reduced after the C. oil (300 mg/kg) treatment (P<0·05; Table 2), suggesting the antioxidant property of C. oil. However, C. oil (30 and 100 mg/kg) did not influence plasma MDA. In order to evaluate the liver function of hamsters on the HC diet alone or with C. oil, we measured the serum level of alanine aminotransferase and aspartate aminotransferase. The HC diet-fed hamsters showed increased alanine aminotransferase and aspartate aminotransferase, signifying liver dysfunction (P<0·01), which
was reversed by the C. oil (100 and 300 mg/kg) treatment ($P<0.05$ and $P<0.001$, respectively; Table 2).

**Curcuma oil attenuates hyperlipidaemia-induced platelet activation**

Platelet activation under hyperlipidaemia was observed in HC diet-fed hamsters (Fig. 2). Therefore, the anti-platelet efficacy of C. oil was assessed against HC diet-induced platelet activation in hyperlipidaemic hamsters. Collagen-, ADP-, and AA-induced aggregation in the whole blood was significantly increased in HC diet-fed hamsters ($P<0.001$; 48, 61 and 53%, respectively; Fig. 2(a)) when compared with the animals fed with the chow diet alone (26, 28 and 22%). The C. oil (300 mg/kg) treatment significantly attenuated ADP- (56%, $P<0.01$), collagen- (62%, $P<0.001$) and AA (47%, $P<0.001$)-induced aggregation. Collagen-induced aggregation was also attenuated with C. oil (100 mg/kg, $P<0.01$); however, the lower dose of C. oil (30 mg/kg) did not show any effect on platelet activation (Fig. 2(a)). Static platelet adhesion was also performed on the collagen- or fibrinogen-coated surface using the platelets from the HC diet-fed hamsters with or without the C. oil treatment. The platelets from the HC diet-fed hamsters adhered more on the collagen- or fibrinogen-coated surface than those from the chow diet-fed group ($P<0.001$ and $P<0.01$, respectively). The C. oil (100 and 300 mg/kg) treatment resulted in a lower number of adhered platelets ($P<0.05$ and $P<0.01$, respectively; Fig. 2(b)). Similar to its anti-platelet effect in hyperlipidaemia, the C. oil treatment also diminished ADP-, collagen- and AA-induced platelet activation and adhesion on the collagen- or fibrinogen-coated surface in chow diet-fed hamsters (data not shown). To assess the effect of C. oil on platelet signal transduction, we conducted platelet protein tyrosine phosphorylation following collagen stimulation. In the present study, the HC diet alone exhibited increased tyrosine phosphorylation of multiple platelet proteins ranging from approximately 120, approximately 70 and approximately 60–55 kDa (Fig. 2(c)), which was moderately enhanced on collagen (5 µg/ml) stimulation. The C. oil (300 mg/kg) treatment in hamsters attenuated tyrosine phosphorylation of platelet proteins (Fig. 2(c)). The property of C. oil to prevent protein tyrosine phosphorylation correlated with its potency to inhibit platelet aggregation.

**Protective effect of curcuma oil on hyperlipidaemia-induced endothelial dysfunction**

Endothelial dysfunction along with the lipid-laden aorta is a frequent observation under hyperlipidaemic conditions. In this regard, we tested the effect of C. oil on endothelial relaxation and eNOS mRNA transcript, together with the effect on aortic lipid accumulation and CD68 mRNA expression. A significant reduction in acetylcholine-induced endothelial relaxation and eNOS expression was observed in the aorta from the HC diet-fed hamsters ($P<0.001$; Fig. 3(a) and (b)). The C. oil (300 mg/kg) treatment in HC diet-fed hamsters restored acetylcholine-induced relaxation ($P<0.001$; Fig. 3(a)) and aortic eNOS mRNA expression ($P<0.001$; Fig. 3(b)). Similarly, the ezetimibe treatment also normalised endothelial relaxation and the eNOS mRNA transcript ($P<0.001$; Fig. 3(a) and (b)).

Enhanced aortic cholesterol and the CD68 mRNA transcript was found in hamsters on the HC diet ($P<0.001$; Fig. 3(c)). The C. oil (300 mg/kg) treatment significantly attenuated cholesterol accumulation in the aortic wall ($P<0.001$; Fig. 3(c)) and reduced CD68 mRNA transcript ($P<0.01$; Fig. 3(b)).

![Fig. 2. Curcuma oil (C. oil) inhibits hyperlipidaemia-induced platelet activation and protein tyrosine phosphorylation. (a) ADP-, collagen- and arachidonic acid-induced whole blood aggregation. (b) Platelet adhesion on the collagen- or fibrinogen-coated surface. (c) Protein tyrosine phosphorylation in the platelets from the Chow diet (□)-fed animals ($P<0.001$; one-way ANOVA). Mean values were significantly different from the chow diet (□)-fed animals ($P<0.001$; one-way ANOVA): † $P<0.05$, †† $P<0.01$. HC + C. oil (30 mg/kg) □, HC + C. oil (100 mg/kg) △, HC + C. oil (300 mg/kg).](https://www.cambridge.org/core/terms). IP address: 54.70.40.11, on 06 Feb 2020 at 22:42:02. Downloaded from https://www.cambridge.org/core. Subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms.
Curcuma oil ameliorates endothelial dysfunction and attenuates aortic cholesterol and macrophage accumulation. (a) Dose–response of acetylcholine (Ach) (3nM–3µM)-induced relaxation in the phenylephrine (1µM)-pre-contracted aortic vessels. Ch.; Chow; HC, high cholesterol (HC); HC + C. oil (300 mg/kg). (b) Aortic endothelial NO synthase (eNOS) expression. (c) Aortic cholesterol. (d) Aortic CD68 mRNA expression. Chow; HC; HC + C. oil (300 mg/kg); HC + ezetimibe (1mg/kg). TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester. Values are means (n=10), with their standard errors represented by vertical bars. Mean values were significantly different from the chow diet-fed animals (one-way ANOVA): **P<0.01, ***P<0.001; Fig. 3(d)). Ezetimibe also prevented aortic cholesterol and macrophage accumulation (**P<0.001; Fig. 3(d)). In agreement with C. oil-induced anti-hyperlipidaemic effect, the mRNA expression of various regulating genes involved in cholesterol homeostasis were up-regulated in the C. oil-treated group. C. oil in chow diet-fed animals also prevented aortic lipid accumulation, as there were significant decreases in the levels of TC (P<0.05), FC (P<0.01) and cholesteryl esters (P<0.05) (Fig. 3(c)). In agreement with C. oil-induced reduction in aortic lipids, HC diet-induced aortic CD68 expression was also reduced in C. oil (300 mg/kg)-treated animals (P<0.001; Fig. 3(d)). Ezetimibe also prevented aortic cholesterol accumulation (P<0.01) and CD68 (P<0.001) expression (Fig. 3(c) and (d)). However, no effect on vascular dysfunction and aortic lipid accumulation was observed with C. oil at the doses of 30 and 100 mg/kg (data not shown).

Curcuma oil exerts its anti-hyperlipidaemic effect by regulating genes involved in cholesterol homeostasis

To explore the possible mechanism involved in the lipid-lowering effect of C. oil, the mRNA expression of various genes from the liver and small intestine (jejunum) engaged in cholesterol homeostasis was examined.

In the chow diet-fed animals, C. oil (300 mg/kg) showed increased hepatic mRNA expression of PPARα (3-fold, P<0.01) and its target gene LPL (3-fold, P<0.05). Furthermore, we observed reduced mRNA expression of SREBP-2 (26-fold, P<0.001) and HMGCR (6-fold, P<0.05), suggesting that the C. oil-induced anti-hyperlipidaemic effect seems to be mediated by PPARα and its target genes (Fig. 4(a)). However, hepatic expression of LDLR was unchanged in the C. oil-treated group. C. oil in chow diet-fed animals also up-regulated LXRA (5-fold, P<0.01) and its target genes CYP7A1 (3-fold, P<0.05), ABCA1 (2-fold, P<0.05), ABCG5 (4-fold, P<0.05) and ABCG8 (3-fold, P<0.01) that were invloved in hepatic cholesterol catabolism and efflux, respectively (Fig. 4(b)).

The HC diet itself decreased the hepatic expression of PPARα (3-fold, P<0.05), SREBP-2 (12-fold, P<0.001), HMGCR (4-fold, P<0.05) and LDLR (7-fold, P<0.01), although the hepatic expression of LPL remained unchanged (Fig. 4(a)). In addition, the HC diet suppressed hepatic CYP7A1 (3-fold) and up-regulated ABCA1 (2-fold), ABCG5 (2-fold) and ABCG8 (2-fold, P<0.05; Fig. 4(b)). The C. oil (300 mg/kg) treatment in HC diet-fed hamsters increased hepatic mRNA expression of PPARα (20-fold, P<0.001) and LPL (5-fold, P<0.05). Importantly, the C. oil treatment attenuated the decrease in LDLR expression in the HC diet group. However, the mRNA transcript of SREBP-2 and HMGCR remained unchanged (Fig. 4(a)). Hepatic LXRA (6-fold, P<0.01), CYP7A1 (4-fold, P<0.01), ABCA1 (4-fold, P<0.05), ABCG5 (5-fold, P<0.05) and ABCG8 (4-fold, P<0.05) were up-regulated with the C. oil treatment in HC diet-fed hamsters (Fig. 4(b)).

In order to ascertain whether the lipid-lowering effect of C. oil involves the genes regulating cholesterol absorption and biliary cholesterol excretion, we evaluated jejunal mRNA expression of NPC1L1, ABCA1, ABCG5 and ABCG8 with or without C. oil (300 mg/kg) in both chow diet- and HC diet-fed animals. The C. oil-treated hamsters showed increased mRNA expression of ABCA1 (2- and 4-fold, P<0.05), ABCG5 (2- and 4-fold, P<0.05) and ABCG8 (2- and 4-fold, P<0.05) in both chow diet- and HC diet-fed groups, respectively (Fig. 4(c)). Moreover, C. oil repressed jejunal NPC1L1 mRNA expression.
expression (19- and 11-fold, \( P < 0.01 \) and \( P < 0.05 \)) in chow diet- and HC diet-fed hamsters, respectively (Fig. 4(c)).

**Discussion**

The experimental findings of the present study revealed that C. oil demonstrated an anti-hyperlipidaemic effect accompanied with improved vascular relaxation, reduced platelet activation and oxidative stress. The anti-hyperlipidaemic effect of C. oil seems to be mediated by the modulation of PPAR\( \alpha \), LXR\( \alpha \) and associated genes that are involved in lipid metabolism and efflux. To the best of our knowledge, this is the first report demonstrating the anti-hyperlipidaemic effect of C. oil that involves PPAR\( \alpha \) and LXR\( \alpha \) activation. As reported earlier, C. oil is mainly comprised of ar-d-turmerone, \( \alpha / \beta \)-turmerone and curlone\(^{2,5} \). In *vivo* pharmacokinetic studies have revealed that oral bioavailability and plasma elimination half-life of ar-turmerone was considerably higher than \( \alpha / \beta \)-turmerone and curlone\(^{5} \). Aromatic turmerones have been documented for their anti-platelet\(^{25} \) and
anti-proliferative\(^{(10)}\) effects; however, very limited or no information is available on the physiological effect of curcule.

In the present study, the effect of C. oil was evaluated in golden Syrian hamsters due to their appropriateness for such studies\(^{(17)}\). We used low, medium and high doses of C. oil (30, 100 and 300 mg/kg, respectively) for assessing the dose-dependent anti-hyperlipidaemic and possible anti-atherogenic effects of C. oil, if any. The low dose of C. oil was ineffective but changes and trends that appeared at 100 mg/kg became more profound at 300 mg/kg. The commonly used body surface area-based dose calculation\(^{(20)}\) indicates that C. oil at 100 and 300 mg/kg in hamsters will be equivalent to about 800 mg and 2.4 g/person per d, respectively, for an adult human\(^{(26)}\). However, it is difficult to translate the exact dose for human use from animal studies, and this has to be done with extreme caution\(^{(26)}\). Previously, turmeric oil (600 mg/d) with turmeric (3 g/d) has been shown to exert a beneficial effect in patients suffering from oral submucous fibrosis\(^{(27)}\).

More importantly, in a previous human study, 600 mg and 1 g/d of turmeric oil for 1 and 3 months, respectively, were found to be safe on haematological, renal and hepatotoxicity parameters\(^{(28)}\). Also, in the present study, C. oil did not exhibit hepatotoxicity at the highest dose (300 mg/kg), and, in fact, it had beneficial effects as reflected by the improvement in liver function test and oxidative stress. Diet surveys in the Asian population showed that regular dietary intake of turmeric for a longer duration was associated with less incidence of cancer and improved cognitive function in those regions\(^{(29,30)}\).

However, no proven correlation has been established by conducting controlled trials. It is therefore quite possible that a regular intake of C. oil in humans at a similar or lower dose for a longer duration might produce a therapeutic benefit against hyperlipidaemia and associated complications. However, a long-term study with lower doses in animals and detailed toxicity and safety evaluations with C. oil need to be carried out before its translation for human use.

Corroborating a previous report in rats\(^{(31)}\), C. oil also inhibited hyperlipidaemia-induced platelet activation and tyrosine phosphorylation in hamsters. The protective effect of C. oil on the vascular wall might be due to its anti-platelet, lipid-lowering, antioxidant or anti-inflammatory activities.

Based on the above results, the 300 mg/kg per d dose regimen was selected for mechanistic evaluations. Similar to PPAR\(\alpha\) activators\(^{(31–33)}\) and NPC1L1 inhibitors\(^{(34,35)}\), the plasma lipid-lowering effect of C. oil was accompanied with reduced aortic and liver lipid accumulation. Furthermore, C. oil reduced aortic macrophage infiltration, recovered vascular dysfunction and normalised eNOS expression. It has been previously shown that the positive regulation of PPAR\(\alpha\) and LXRs reduces aortic lipid accumulation and atherosclerosis in dyslipidaemic hamsters\(^{(32,33)}\). Moreover, PPAR\(\alpha\) activators have been reported to enhance eNOS protein expression by stabilising eNOS mRNA in endothelial cells\(^{(36)}\). Thus, it is likely that the C. oil-mediated anti-hyperlipidaemic effect and improved vascular function involve the activation of PPAR\(\alpha\), LXRs and their target genes.

To delineate the possible mechanism of C. oil-induced lipid lowering, we assessed the effect of C. oil on the transcriptional regulation of different enterohepatic genes involved in cholesterol metabolism and efflux. Since most of the lipid-related genes are up- or down-regulated with a diet rich in cholesterol and fat\(^{(37)}\), we therefore evaluated the effect of C. oil in both chow diet- and HC diet-fed hamsters.

The liver and gut are considered as two major organs working in tandem to maintain cholesterol homeostasis in the body\(^{(38,39)}\). While the liver is involved in de novo cholesterol synthesis, catabolism and its release via the modulation of PPAR\(\alpha\), LXRs and their target genes\(^{(15)}\), the gut plays a pivotal role in cholesterol absorption via genes such as NPC1L1\(^{(39,35)}\).

The major lipid-related target genes of PPAR\(\alpha\) are LPL and SREBP-2, while that of LXRs are CYP7A1, ABCA1, ABCG5 and ABCG8\(^{(15,30)}\). Hepatic LPL and PPAR\(\alpha\) mRNA expression were up-regulated by the C. oil treatment. LPL hydrolyses TAG-rich lipoproteins and produces hypolipidaemic and anti-atherogenic effects\(^{(15,40)}\). SREBP-2, another target gene of PPAR\(\alpha\), is primarily involved in cholesterol synthesis\(^{(41)}\) and is also known to regulate the hepatic expression of HMGCR and LDLR\(^{(31,42)}\), the key proteins involved in liver cholesterol enrichment. Concomitant suppression of hepatic SREBP-2 and HMGCR by C. oil was observed in chow diet-fed hamsters. Although hepatic LDLR expression was unaffected in chow diet-fed hamsters, C. oil restored the HC diet-suppressed LDLR expression in HC diet-fed hamsters. This difference in the results could be due to less hepatic cholesterol in the C. oil-treated group that increased hepatic LDLR expression. Since the cholesterol-rich diet alone diminished hepatic SREBP-2 and HMGCR, as also reported earlier\(^{(57)}\), further reductions in SREBP-2 and HMGCR were not observed or needed in the C. oil-treated group.

LXR are recognised as sterol sensors, which transcriptionally regulate an array of genes engaged in cholesterol homeostasis and reverse cholesterol transport\(^{(30)}\). LXRs expressed chiefly in enterohepatic tissues\(^{(30)}\), and their activation in dyslipidaemic hamsters led to an increase in macrophage-to-faeces reverse cholesterol transport\(^{(30)}\). Consistent with these lines of observation, we found that C. oil amplified the hepatic expression of LXRs, along with CYP7A1, a rate-limiting enzyme that converts cholesterol into bile acids in the liver.

Enterohepatic expression of ABC transporters, i.e. ABCA1, ABCG5 and ABCG8, was up-regulated after the C. oil treatment. The overexpression of ABCG5/8 in the liver and small intestine led to less intestinal cholesterol absorption and enhanced faecal neutral sterol excretion\(^{(43)}\). Enterohepatic ABCA1 is involved in HDL biogenesis and maintaining mature HDL in a PPAR/PPAR\(\alpha\)-dependent manner\(^{(44)}\). This might explain the HDL-C-elevating effect of C. oil observed in the present study since we also observed enhanced expression of enterohepatic ABCA1.

NPC1L1, a key regulator of intestinal cholesterol absorption\(^{(55)}\), was down-regulated upon C. oil treatment. Moreover, NPC1L1 is also known to be regulated in a PPAR\(\alpha\)- and LXRs-dependent manner\(^{(14,45)}\), thus their involvement in the repression of jejunal NPC1L1 by C. oil seems to be plausible.

From the present study, it can be concluded that C. oil exerts an anti-hyperlipidaemic effect and ameliorates lipid-induced oxidative stress, platelet activation and vascular
dysfunction. The anti-hyperlipidaemic effect of C. oil seems to be mediated by PPARα, LXRs and associated enterohepatic genes engaged in cholesterol absorption, metabolism and transport. The pathways modulating lipid metabolism in both humans and hamsters are quite similar. Nuclear receptors (i.e. PPAR and LXR) in conjunction with LPL, CYP7A1 and ABCA1 modulate lipid metabolism and efflux in both human subjects and hamsters. The PPARα activator fenofibrate exerts a protective effect in human subjects and hamsters by modulating these genes<sup>[46–49]</sup>. Since, in the present study, C. oil affects these genes in a similar manner, it is quite likely that C. oil might exert anti-hyperlipidaemic effects in humans by similar mechanisms.

By modulating enterohepatic ABCG5/G8 and jejunal NPC1L1, C. oil may improve dyslipidaemia by favouring biliary and faecal cholesterol excretion. These changes may have a positive impact on macrophage-to-faeces reverse cholesterol transport. This further emphasises the antiatherogenic potential of C. oil. However, more studies are needed to validate the proposed hypothesis. The present paper is CSIR-CDRI communication no. 8348.

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