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Combination of spices and herbal extract restores macrophage foam cell migration and abrogates the athero-inflammatory signalling cascade of atherogenesis

Chetan Nimgulkar [a], Sudip Ghosh [b], Anand B. Sankar [b], Kumar P. Uday [c], M.V. Surekha [c], P. Madhusudhanachary [c], B.R. Annapurna [a], P. Raghu [d], Dinesh Kumar Bharatraj [a,*]

[a] Food and Drug Toxicology Research Centre, National Institute of Nutrition, (ICMR), Jamai Osmania, Hyderabad 500 007, India
[b] Molecular Biology Unit, National Institute of Nutrition, (ICMR), Jamai Osmania, Hyderabad 500 007, India
[c] Pathology Division, National Institute of Nutrition, (ICMR), Jamai Osmania, Hyderabad 500 007, India
[d] Biophysics Division, National Institute of Nutrition, (ICMR), Jamai Osmania, Hyderabad 500 007, India

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ABSTRACT

The trapping of lipid-laden macrophages in the arterial intima is a critical but reversible step in atherogenesis. However, information about possible treatments for this condition is lacking. Here, we hypothesized that combining the polyphenol-rich fractions (PHC) of commonly consumed spices (*Allium sativum* L (Liliaceae), *Zingiber officinale* R (Zingiberaceae), *Curcuma longa* L (Zingiberaceae)) and herbs (*Terminalia arjuna* (R) W & A (Combretaceae) and *Cyperus rotundus* L (Cyperaceae)) prevents foam cell formation and atherogenesis. Using an in vitro foam cell formation assay, we found that PHC significantly inhibited lipid-laden macrophage foam cell formation compared to the depleted polyphenol fraction of PHC (F-PHC). We further observed that PHC attenuated the LDL and LPS induced CD36, p-FAK and PPAR-γ protein expression in macrophages and increased their migration. NK-κB–DNA interaction, TNF-α, ROS generation, and MMP9 and MMP2 protein expression were suppressed in PHC-treated macrophages. The anti-atherosclerotic activity of PHC was investigated in a high fat- and cholesterol-fed rabbit model. The inhibition of foam cell deposition within the aortic intima and atheroma formation confirmed the atheroprotective activity of PHC. Therefore, we conclude that the armoury of polyphenols in PHC attenuates the CD36 signalling cascade-mediated foam cell formation, enhances the migration of these cells and prevents atherogenesis.

1. Introduction

Cardiovascular diseases are the leading causes of death worldwide, and atherosclerosis is the most important factor that contributes to the aetiology and pathology of CVDs [1]. Atherosclerotic intimal thickening is a result of the accumulation of lipid-laden macrophages and lymphocytes due to the uptake of 'invading' oxidized pathogenic lipoproteins in the arterial wall [2,3]. Macrophages have evolved to protect the body from infection via the phagocytosis of pathogens. However, the same properties of macrophages are responsible for the uptake of oxidized lipoproteins and the release of reactive oxygen species (ROS) and other immune mediators. The net effect of this process is foam cell formation and atherogenesis [4]. Oxidized LDL (oxLDL) possesses a high affinity towards macrophage CD36 scavenger receptors, which are also known as FAT (fatty acid translocase), that contribute to lipid accumulation within the cells [5]. CD36 mediates cell-specific responses, such as the inhibition of angiogenesis, promotes endothelial cell apoptosis, and stimulates inflammatory responses (activation nuclear factor-kappa-light-chain-enhancer of activated B cells [NF-κB]) and phagocytosis [6]. The uptake of bioactive lipids that directly target peroxisome proliferator-activated receptor gamma (PPAR-γ) and trigger the hyper-phosphorylation of focal adhesion kinase (FAK) results in macrophage foam-cell formation and entrapment in the neointima. The entrapped foam cell within the intima subsequently initiates and propagates atheroinflammatory lesions [1,7].

As a signalling molecule, CD36 plays significant roles in atherogenic processes, including foam cell formation, the release of inflammatory mediators, macrophage trapping and thrombosis. Therefore, targeting the CD36 receptor signalling cascades, ROS generation and LDL oxidation is an attractive option for the treatment of atherosclerosis. However, information on anti-atherosclerotic therapeutic leads from natural sources to target CD36 signalling pathogenic pathways is lacking.

Many spices show anti-hyperlipidemic, anti-inflammatory and antioxidant activities and are less toxic than allopathic medicines [8]. Hence, the combination of spices and herbs can be translated into an anti-atherosclerotic functional food via reverse pharmacological trans-

* Corresponding author at: FDTRC, NIN (ICMR), Jamai Osmania, Hyderabad 500 007, India.
E-mail address: dineshantioxidant@gmail.com (D.K. Bharatraj).

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disciplinary exploratory studies [9]. Based on the traditional and current ethnopharmacological literature, specific spices and herbs were selected for their anti-inflammatory, anti-oxidant and anti-hyperlipidemic properties. Garlic (*Allium sativum* Linn.) (Liliaceae) is a potent anti-hyperlipidemic spice that inhibits cholesterol synthesis and LDL oxidation. Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) exerts potent hypolipidemic and anti-oxidant effects by scavenging free radicals. Turmeric (*Curcuma longa* Linn., Zingiberaceae) is known to possess anti-inflammatory properties that attenuate the NF-κB inflammatory signalling pathway [10]. The bark of *Terminalia arjuna* (Roxb) Wight & Am., (Combretaceae) is known to possess cardiotonic and anti-atherosclerotic properties [11]. *Cyperus rotundus* Linn. (Cyperaceae), a traditional medicine, possesses anti-inflammatory activity [12]. The polyphenols present in these spices and herbs exert a pleiotropic effect in terms of the prevention of atherosclerosis. However, the pharmacological potential of combined polyphenols remains to be translated [13].

These spices and herbs are being widely used individually in the form of dried powders and extracts as dietary supplements for the treatment of atherogenesis [8]. However, these spices or herbs are insufficient to counteract multi-aetiological foam cell formation and atherogenesis when used individually [2]. Therefore, we hypothesized that the combination of these spices and herbal extracts can strengthen the polyphenol armoury that will target the multiple aetiologies of atherogenesis.

2. Methods and methods

2.1. Extraction of test material

Dried test samples of *A. sativum* Linn., *Z. officinale* Roscoe., *C. longa* Linn., *T. arjuna* (Roxb) Wight & Am. and *C. rotundus* Linn were obtained from the *Dabur* Research and Development Centre, Ghaziabad India free of charge. The qualitative compliance of the herbal dry powder was confirmed according to the Indian Pharmacopoeia (IP) and WHO monographs (WHO). The test materials were extracted in 50% ethanolic (v/v) solvent. Briefly, 2 kg of each sample was individually macerated and vortexed in 5 L of 50% ethanol at room temperature for 20 h. The extract was centrifuged at 200 *g* for 10 min, and the clarified supernatant was decanted in a graduated tube. The extraction step was repeated thrice. All extracts were pooled, and their volumes were reduced by up to 75% under a vacuum evaporator (Buchi Rotavapor R205) at <45 °C. The extracts were finally dried by lyophilization. The dried individual extracts were stored at −20 °C until further use. The dried individual extracts (50 g) of *A. sativum*, *Z. officinale*, *C. longa*, *T. arjuna* and *C. rotundus* were combined and mixed at equal ratios to obtain a polyphenol-rich combination, which is referred to as "PHC" throughout this study.

2.2. Filtration of PHC through column

To probe the effect of a combination of polyphenols on foam cell formation, the PHC was filtered through Oasis HLB (1 cc vac cartridge, 30 mg sorbent per cartridge, 30-μm particle size, [WAT094225]), which is a polymeric reversed-phase sorbent. Because the Oasis HLB sorbent is water wettable, it features a higher retention capability and excellent recoveries. The filtration of PHC with this syringe-barrel-type cartridge extracts a wide range of acidic, basic, and neutral compounds. The filtered fraction of PHC is called filtered PHC (F-PHC).

2.3. Determination of total polyphenol content

The total polyphenol contents (TPC) of PHC and F-PHC were determined by spectrophotometry using gallic acid as a standard according to the method described by the International Organization for Standardization (ISO) 14502-1 [14]. The TPC is expressed as gallic acid equivalents (GAE) in g/g material.

2.4. Determination of total flavonoids content

The total flavonoid contents (TFC) of PHC and F-PHC were estimated as described by Bekir [15]. Quercetin was used as the reference compound to produce the standard curve; the results are expressed as mg of quercetin equivalents (QE)/g of dw.

2.5. Cell culture

Mouse macrophage J774A.1 cells were obtained from the National Centre for Cell Science (NCCS) Pune, India and cultured in a humidified atmosphere of 95% air–5% carbon dioxide at 37 °C using Dulbecco's modified Eagle's medium (DMEM) supplemented with 4 mM L-glutamine, 10% heat-inactivated foetal bovine serum and 1% penicillin–streptomycin. The IC_{50} of PHC and F-PHC were found to be 865.1 μg/mL and 1286 μg/mL, respectively, for J774A.1 mouse macrophages using a MTT assay (unpublished data). One tenth of the PHC and F-PHC IC_{50} doses were used to measure the in vitro foam cell formation activity.

2.6. Foam cell formation assay

To demonstrate the effect of polyphenols on macrophage foam cell formation, we modified the lipid uptake assays described by Park et al. [16]. Briefly, the J774A.1 cells (2×10^5 cells/well) were seeded in a 6-well plate and incubated with PHC and F-PHC (1 to 200 μg/mL) for 12 h at 37 °C. Following incubation, the cells were treated with LPS (5 μg/mL) and LDL (50 μg/mL) for 24 h. The cells were fixed with 6% paraformaldehyde, stained with Oil-Red O and counter-stained with haematoxylin. The lipids were then measured spectrophotometrically (520 nm) following isopropanol extraction, and a morphological evaluation was carried out via microscopy.

2.7. Quantification of polyphenols, and flavonoids from PHC

PHC was found to be enriched in TPC and TFP, and it inhibited foam cell formation better than F-PHC. Hence, the polyphenols were quantified, and further experiments were performed with PHC only. Polyphenols (phenolic acids, iso-flavones, flavones, flavonols, flavanols and flavan-3-ols) from PHC were extracted using 90% methanol containing 0.5% acetic acid and measured by HPLC [17]. The mobile phase was run with a combination of — 'A' (50 mM sodium dihydrogen phosphate dihydrate buffer: methanol) (9:1) and 'B' (50 mM sodium dihydrogen phosphate dihydrate buffer: 70% Methanol) (3:7) in gradient manner at a flow rate of 0.47 mL/min for 20 min. A Dionex PA2 RSLC, (Ultimate 3000) 120 Å C18 (100 mm × 2.1 mm) 2.1-μm column was used. The sample was filtered through a 0.2-μm filter for injection, and the absorbances were measured at 250, 280, 320, and 370 nm.

2.8. Western blot analysis

Mouse J774A.1 macrophages were incubated with PHC for 12 h and exposed to LPS (5 μg/mL) and LDL (50 μg/mL) for 24 h. The cells were then lysed in a solution of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate. The clarified lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with antibodies against CD36, PPAR-γ and phospho-FAK. After chemiluminescence detection, the membranes were stripped with stripping buffer and re-probed with antibodies against β-actin and FAK for normalization. The band intensities were quantified using ImageJ (http://rsbweb.nih.gov/ij/).