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The Protective Effect of *Crataegus aronia* Against High-Fat Diet-Induced Vascular Inflammation in Rats Entails Inhibition of the NLRP-3 Inflammasome Pathway

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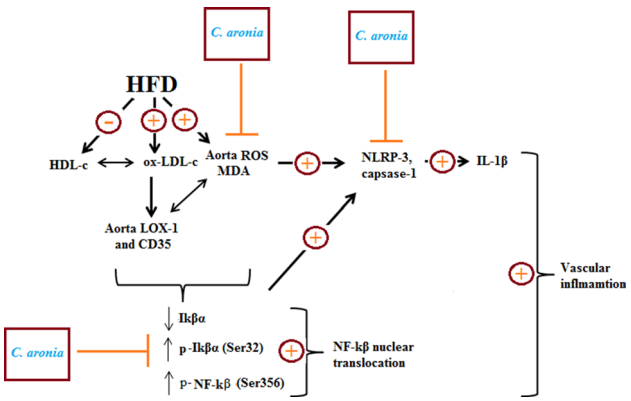
Abstract

This study investigated whether the whole-plant aqueous extract of *Crataegus aronia* (*C. aronia*) could protect against or alleviate high-fat diet (HFD)-induced aortic vascular inflammation in rats by inhibiting the NLRP-3 inflammasome pathway and examined some mechanisms of action with respect to its antioxidant and hypolipidemic effects. Adult male Wistar rats were divided into five groups ($n=6$/each): standard diet (10% fat) fed to control rats, control + *C. aronia* (200 mg/kg), HFD (40% fat), HFD + *C. aronia*, and HFD post-treated with *C. aronia*. The HFD was fed for 8 weeks and *C. aronia* was administered orally for 4 weeks. In addition, isolated macrophages from control rats were pre-incubated with two doses of *C. aronia* (25 and 50 µg/mL) with or without lipopolysaccharide (LPS) stimulation. Only in HFD-fed rats, co- and post-*C. aronia* therapy lowered circulatory levels of LDL-C and ox-LDL-c and aortic protein levels of LOX-1 and CD36. *C. aronia* also inhibited the nuclear accumulation of NF-κB and lowered protein levels of NLRP-3, caspase-1, and mature IL-1β. In vitro, in the absence of ox-LDL-c, *C. aronia* led to reduced nuclear levels of NF-κB, ROS generation, and protein NLRP-3 levels, in both LPS-stimulated and unstimulated macrophages, in a dose-dependent manner. However, protein levels of LOX-1 were not affected by *C. aronia* in unstimulated cells. In conclusion, *C. aronia* inhibits the NLRP-3 inflammasome pathway, induced by HFD feeding in the aorta of rats, mainly by its hypolipidemic effect and in vitro, in LPS-stimulated macrophages, by its antioxidant effect.

Graphic Abstract



Keywords Vascular inflammation · *C. aronia* · ox-LDL-c · NLRP-3 inflammasome

Abbreviations

CARD	Caspase recruitment domain
C. aronia	*Crataegus aronia*
CHOL	Cholesterol

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HDL-c	High-density lipoprotein cholesterol
ECs	Endothelial cells
HFD	High-fat diet
IL-6	Interleukin-6
IL-1β	Interleukin 1β
IκBα	Inhibitory factor kappa B-α subunit
IKK	IκB kinase
LDL-c	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LOX-1	Oxidized LDL receptor
MDA	Malondialdehyde
NF-kB	Nuclear factor kappa B
ox-LDL-c	Oxidized low-density lipoprotein cholesterol
ROS	Reactive oxygen species
STD	Standard diet
TNF-α	Tumor necrosis factor-α
VSMs	Vascular smooth muscles

Introduction

Local aseptic vascular inflammation plays an important role in all phases of atherosclerosis from its initiation to its clinical manifestations, including plaque rupture [1]. The accumulation of intracellular oxidized low-density lipoprotein cholesterol (ox-LDL-c) has been found to be the initial step of atherogenesis at the arterial walls, which triggers all other cellular manifestations, including activation of endothelial cells (ECs), migration of smooth muscle cells (SMCs), overexpression of cell adhesion molecules, recruitment of monocyte/lymphocyte, and subsequent sustained release of the pro-inflammatory cytokines [1–3]. In the arterial wall, these pro-inflammatory cytokines can also re-activate the ECs, SMCs, and themselves to foster cytokine production, leading to a self-perpetuating inflammatory process that becomes, with time, less dependent on the presence of ox-LDL-c, thus leading to chronic inflammation [3].

However, the exact mechanisms linking hyperlipidemia with vascular inflammation in the process of atherosclerosis remain elusive. Currently, the inhibition of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP-3) inflammasome, or the inhibition of interleukin 1β in the arterial walls were shown to be promising strategies to slow down and prevent the development of atherosclerosis [4, 5]. This is supported by the presence of all components of the NLRP-3 inflammasome in the cells participating in vascular inflammation, including ECs, SMCs, monocytes, macrophages, dendritic cell, and T lymphocytes [4]. Furthermore, the activation of the NLRP-3 inflammasome can be induced in the vascular cells by ox-LDL-c and cholesterol (CHOL) crystals [4]. In addition, increased expression levels of NLRP-3 inflammasome were observed in the aortas of patients with coronary atherosclerosis and were highly correlated with circulatory levels of ox-LDL-c [6, 7] whereas pharmacological inhibition of this inflammasome reduced atherosclerosis and stabilized plaques in ApoE-deficient mice [8].

In accordance, anti-cytokine therapy became one of the most promising options for the early prevention of systemic inflammation and subclinical atherosclerosis [9, 10]. Indeed, pharmacological inhibition or genetic deletion of IL-1β prevented the increase in interleukin-6 (IL-6), oriented tissue macrophages to an anti-inflammatory phenotype, and reduced atherosclerotic lesions in an atherosclerotic mouse model [11, 12]. However, the use of anti-inflammatory dietary supplements derived from medicinal plants may provide an alternative approach to the prevention of subclinical atherosclerosis relative to synthetic drugs [9].

Hawthorn (*Crataegus*) (C.) is a plant that belongs to the Rosaceae family and is currently used worldwide to treat several disease conditions, including hyperlipidemia, hyperglycemia, cardiovascular disorders, atherosclerosis, and hypertension [13]. Interestingly, all Hawthorn parts, including fruits, flowers, and leaves are used in the therapy towing of their antioxidant, hypolipidemic, and anti-inflammatory effects, which are attributed to their high polyphenol content [13–17]. There are over 100 different Hawthorn species worldwide [13]. *Crataegus monogyna* and *C. laevigata* are the most common species used for medication and extraction in Europe [13], whereas *C. pinnatifida* and *C. scabrifolia* are most commonly used in China [17, 18].

Crataegus oxyacantha, *C. monogyna*, and *C. laevigata* are the best-characterized Hawthorn species with cardiovascular effects [13, 19–25]. These species aid in improving cardiac contractility and inducing endothelium-dependent vasodilation via several mechanisms, such as increasing cardiac intracellular Ca^{2+} levels, inhibition of the cAMP phosphodiesterase activity, stimulating nitric oxide (NO) synthesis and release, and inhibition of the angiotensin-converting enzyme (ACE) [13, 19–25]. However, *C. oxyacantha* and *C. meyeri* exert an anti-arrhythmic effect mediated by blocking the repolarization of K^+ currents [26–28]. On the other hand, *C. oxyacantha* fruits prevented hyperlipidemia and hypercholesterolemia in rats fed with an atherogenic diet by upregulating hepatic LDL-c receptors, stimulating the degradation of CHOL into bile acids, and increasing bile acid excretion [29]. Similarly, dried fruits of *C. pinnatifida* prevented hypercholesterolemia and reduced accumulation of CHOL in the aorta of high CHOL-fed rabbits through downregulation of intestinal acyl-coenzyme A:cholesterol acyltransferase activity (ACAT) and subsequent inhibition of CHOL absorption [30]. Furthermore, hepatic accumulation was prevented in high-fat diet-fed mice by downregulating the expression of sterol regulatory element binding protein-1c (SREBP-1c) and fatty acid synthase (FAS) [31]. In addition, extracts prepared from *C. oxyacantha* and *C.*

pinnatifida have ROS-scavenging activities that protected LDL-c from oxidation and exerted anti-inflammatory effects, independent of their hypolipidemic or antioxidant effects [32–34].

On the other hand, *C. aronia*, syn. Azarolus (L.) is the most predominant hawthorn species found in the mountains of the Mediterranean basin and in Arabian countries [35]. Similar to their sister species, *C. aronia* exhibits potent antioxidant, hypolipidemic, anti-inflammatory, and antioxidant effects in various animal models. In addition, we have previously shown that aqueous extract of *C. aronia* is well tolerated (up to 2000 mg/kg) in rats [36] and exerts potent negative chronotropic, positive inotropic, and hypotensive effects, through stimulation of the muscarinic receptor (M2) and NO release [37]. Interestingly, most of the studies that investigated the health benefits of *C. aronia* in rats have shown that the maximum effect can be achieved by using its aqueous extract at a final dose of 200 mg/kg [37–41]. Indeed, in a rat model of non-alcoholic fatty liver disease (NAFLD), chronic administration of *C. aronia* at a dose of 200 mg/kg for 4 weeks, inhibited hepatic fat accumulation, lowered circulatory levels of CHOL, triglycerides (TGs), and LDL-c [38]. In the same animal model and at a similar dose, it also protected the testis and restored normal sperm parameters and productive function from oxidative damage due to its hypolipidemic effects and its independent antioxidant potential, mediated by upregulation of the nuclear 2-related factor 2 (Nrf2) and by stimulating the synthesis of superoxide dismutase (SOD) and glutathione (GSH) [39]. The hypolipidemic, hypoglycemic, and anti-inflammatory effects of *C. aronia* have also been demonstrated in a diabetic animal model, where the aqueous extract of this plant aided in increased expression of insulin receptors and inhibition of gluconeogenesis [40]. In addition, *C. aronia* has been shown to scavenge superoxide radicals, stimulate GSH synthesis, and prevent Fe^{2+}-induced lipid peroxidation in rat liver homogenates [35].

Notably, we have recently provided the first evidence that the co- or post-administration of the aqueous extract of *C. aronia* for 4 weeks, at a dose of 200 mg/kg, could ameliorate vascular atherosclerosis in rats, induced by an 8-week high-fat diet (HFD), by decreasing the thickness of the tunica media of the aorta, inhibiting the circulatory levels of LDL-c, ox-LDL-c, and C-reactive protein (CRP), increasing circulatory levels of high-density lipoprotein cholesterol (HDL-c), and downregulation of the aortic expression of tumor necrosis factor-α (TNF-α) and IL-6 [41]. However, despite the above-mentioned studies, the mechanism by which *C. aronia* exerts its anti-inflammatory action is still not well established. Moreover, whether the anti-inflammatory effect is due to the extract activity on inflammation key modulators or just a secondary effect of its hypolipidemic and/or antioxidant activities is still a matter of debate.

Hence, the objective of this study was to examine the impact of co- or post-administration of *C. aronia* on the expression of the NLRP-3 inflammasome and IL-1β in the aorta of a HFD-fed rat model of vascular inflammation and to identify the possible mechanisms behind this effect from the aspects of its antioxidant and hypolipidemic activities.

Materials and Methods

Preparation of the Extract

The aqueous extract of *C. aronia* used in this study was prepared from the aerial parts, including stems, flowers, and leaves, as previously described in our laboratory [36, 37]. The whole plant was purchased from a licensed herbal supplier (Kabatilo Natural products store) in Amman, Kingdom of Jordan, where their records showed that the plant was collected freshly, dried, and preserved for only 20 days. The plant was identified by the Pharmacognosy staff at the College of Pharmacy, King Khalid University based on an available voucher specimen. In brief, the whole plant was grounded into a powder and extracted by maceration using distilled water (1 kg/1 L, w/v) for 3 days at 37 °C. The extract was filtered and evaporated under reduced pressure in a rotary evaporator. The resulting residue (40–50 g) was always stored at 4 °C and used every 3 days to prepare the aqueous extract by dissolving in distilled water to a final concentration of 200 mg/mL that is used in this study.

Experimental Animals

Thirty adult male Wistar rats (120 ± 10 g, 6 weeks old) were obtained from the animal facility at College of Medicine at King Khalid University (KKU) in Abha, Kingdome of Saudi Arabia (KSA). The rats were kept in a room under controlled temperature of 22 ± 2 °C, relative humidity of 55 ± 10%, and a light/dark cycle of 12 h each. All procedures performed in this study were approved by the College of Medicine Ethical Committee at KKU which follows the guidelines of laboratory animal care and use, published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Experimental Design

Adult male Wistar Rats (120 ± 10 g) were classified into 5 groups ($n = 6$/each) as (1) *Control (STD)* control rats fed on a standard diet (STD) (3.8 kcal/g, 10% of energy as fat) for 12 weeks; (2) *Control + C. aronia* rats fed on the STD for the first 8 weeks and then co-received *C. aronia* (200 mg/kg/day) during the last 4 weeks; (3) *HFD* rats fed on a HFD (5.4 kcal/g, 40% of calories as fat) for the first 8 weeks and then fed on the STD for the next 4 weeks; (4) *HFD + C.*

Table 1 Ingredient and nutrient composition of standard (STD) and the high-fat diet (HFD)

Ingredients (g/kg)	STD	HFD
	g/diet	g/diet
Casein	200	270
Starch	551	485
Sucrose	100	100
Beef tallow	44.5	240
Cellulose	250.0	050.0
Vitamin–Mineral Premix	050.0	050.0
DL-Methionine	003.0	003.0
Choline chloride	002.0	002.0
	Energy provided	
Carbohydrates	20%	20%
Protein	70%	40%
Fat	10%	40%
Caloric value (kcal/g)	4.0	5.4

Table 2 Primers used in the quantitate polymerase chain reaction (qPCR)

Gene	Acc. no.	Forward Primer 5′→3′	Reverse primer 3′→5′
TNF-α	NM012675	GCCCAGACCCTCACACTC	CCACTCCAGCTGCTCCTCT
IL-6	NM012589.1	TGTATGAACAGCGATGATG	AGAAGACCAGAGCAGATT
18S RRNA	NM031144.3	ACCGAGCATGGCTACAGCGTCACC	GTGGCCATCTCTTGCTCGGAGTCT

aronia rats fed on a HFD and received a concomitant dose of *C. aronia* (200 mg/kg/day) for the first 8 weeks and then continued on the STD for the next 4 weeks; (5) *HFD then C. aronia*: rats fed on a HFD for the first 8 weeks and then post-treated with *C. aronia* (200 mg/kg/day) for the next 4 weeks. Both diets were prepared in our laboratory; their ingredients and energy content are shown in Table 1. Administration of C. aronia was via gavage. The dose of *C. aronia* used in this study was based on our and other previous studies that have shown hypolipidemic, antioxidant, and anti-inflammatory effects of the aqueous extract of this plant at this dose in HFD-fed rats and NAFLD-induced rat model [37–39, 41]. In addition, the HFD regimen was selected based on our previous findings that showed persistent hyperlipidemia and aortic atherosclerosis in the aorta rats fed on this HFD for 8 consecutive weeks, even after withdrawal of the HFD for the next 8 weeks which can be reversed by co-administration of *C. aronia* for 8 weeks or its post-administration for 4 weeks [38, 41].

Serum and Aorta Collection and Biochemical Measurements

Twelve hours after the last treatment, all rats were fasted overnight and anesthetized using an intra-peritoneal (i.p). bolus of sodium pentobarbital (60 mg/kg). Blood samples were collected for serum separation. Each rat was then killed by cervical dislocation and the abdominal aorta was dissected, washed in ice-cold phosphate buffer saline (PBS, pH 7.4), cut in cross sections into smaller pieces, snap frozen in liquid nitrogen, and stored at − 80 °C for further use. Serum levels of LDL-c and HDL-c were measured using a commercial colorimetric assay kit (Human Diagnostics, Germany). Serum levels of malondialdehyde (MDA) were measured using an assay kit (Cat. No. NWK-MDA01, NWLSS, USA). Serum levels of ox-LDL-c were measured using a rat ELISA kit (Cat. NO. MBS2501477, Mybiosource, CA, USA). Serum levels of IL-1β were determined using a rat ELISA kit (Cat. No. ab100767, Abcam, UK). Total levels of ROS and reactive nitrogen species (RNS) in the aorta homogenates (or in isolated macrophages homogenates) were measured using OxiSelect™ In Vitro ROS/RNS Assay Kit (Cat. NO, STA-347, Cell Biolabs, Inc. San Diego, CA). All procedures were conducted in duplicate ($n = 6$/group) and were performed according to the manufacturer's instructions.

Quantitative Real-Time PCR (qRT-PCR)

This procedure was performed to measure aortic mRNA levels of IL-6, TNF-α, and the housekeeping gene, β-actin. Primer sequences of all these genes were adopted from other studies [42–44] and are shown in Table 2. In brief, total RNA was extracted from frozen aortas using an RNeasy Mini Kit (Cat. No. 74104, Qiagen, Victoria, Australia). The purity and concentration of RNA in all samples were determined using a NanoDrop spectrophotometer (Thermo Fisher, MA, USA). The single-stranded cDNA was synthesized using a Superscript II reverse transcriptase kit using oligo (dT) primers (Cat. NO. 18064014, Thermo Fisher, MA, USA). qPCR run was performed using SsoFast EvaGreen Supermix (Cat. NO. 172-5200, Bio-Rad, Montreal, Canada) in a CFX96 real-time PCR system (Bio-Rad, CA, USA). In every plate, the template cDNA was omitted as a control. All measurements were taken for six samples/group and the mRNA level of each gene was expressed relative to the corresponding β-actin mRNA level. All

procedures were performed in accordance with the manufacturer's instructions.

Peritoneal Macrophage Isolation

This procedure was performed to investigate whether the effect of *C. aronia* on LPS induced inflammation and to determine whether the NLRP-3 inflammasome expression is independent of its hypolipidemic effect. Elicited peritoneal exudates were isolated from control healthy male Wister rats as previously described by Xie et al. [45] with some modifications. In brief, each rat was injected i.p. with 4 mL sterile 4% thioglycollate broth. Four days later, each animal was euthanized by a rapid cervical dislocation to avoid excessive bleeding. The abdominal hair was removed, the skin was swapped with 70% alcohol, and a small incision along the midline was performed with sterile scissors to expose the intact peritoneal wall. Then, 10 mL of ice-cold sterile RPMI 1640 medium was directly injected in the peritoneal cavity. The peritoneal exudate was aspired again and centrifuged at 1000 rpm at 4 °C for 10 min. Each pellet was then treated with 0.5 mL of ammonium–chloride–potassium (ACK) lysis buffer (Cat No. A1049201, Thermo Fisher, Scientific) to remove any red blood cells. Cells were then washed and cultured in tissue culture dishes using the RPMI 1640 media supplemented with 10 mM HEPES, 10% inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin–100 mg/mL streptomycin, under a humidified atmosphere of 5% CO_2. The cells were allowed to adhere for 2 h. Non-attached cells were carefully removed by washing cells four times with Hanks' balanced salt solution and then re-cultured in the same media for the next 24 h. This procedure yielded more than 90% of the adherent cell as macrophages.

Macrophage Treatment

LPS was prepared in the culture media to a final concentration of 100 ng/mL and used for the stimulation of macrophages as previously described [45]. Similarly, the aqueous extract of *C. aronia* was also dissolved in the culture media in a stock solution of 100 µg/mL. The macrophages were seeded in 96-well plates at a density of 2×10^4 cells/well for the next 24 h and were treated as (1) *control* incubated in the culture media without any treatment, (2) *control + C. aronia* incubated with two concentrations of *C. aronia* (25 and 50 µg/mL) for 24 h, (3) *LPS-stimulated* incubated with the media for 24 h and then stimulated with LPS (100 ng/mL) for the next 12 h. (4) *LPS + C. aronia-treated cells* incubated with *C. aronia* at either dose for 24 h and then exposed to LPS for the next 12 h. Our preliminary cell viability data using the MTT assay have shown that *C. aronia* does not induce significant cytotoxicity in macrophages at the tested doses (1–100 µg/mL) (data not shown) as cell viability remained higher than 90%. Similarly, we tested the LPS dose, and in accordance with the other studies it induced a 50% decrease in macrophage viability after 12 h of incubation (data not shown).

Cell Viability Assay

The cell viability was evaluated by the MTT (tetrazolium blue thiazol-3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium) assay, according to the method of Soromou et al. [46]. In brief, after the incubation periods, 20 µL of 5 mg/mL MTT reagent was added to each well and the cells were incubated for an additional 4 h. Then, the media were discarded and the plate was completely dried. Next 100 µL of DMSO was added to each well and the optical density was measured at 570 nm. Cell viability was expressed as a percent of the control. Total ROS species levels in each cultured fraction using OxiSelect™ In Vitro ROS/RNS Assay Kit (Cat. NO, STA-347, Cell Biolabs, Inc. San Diego, CA) according to the manufacturer's instructions and data were presented as a percentage of control macrophages incubated with the media alone.

Preparation of Total Cell Homogenates and Cytoplasmic and Nuclear Extract

The NE-PER Nuclear and Cytoplasmic Extraction kit (Cat No. 78835, Thermo Fisher Scientific) was used to prepare both fractions from frozen aorta samples and cultured macrophages. For total cell homogenate preparation for western blot analysis, frozen aorta (30 mg) or cultured macrophages from all groups were harvested in 0.5 mL RIPA buffer containing 50 mM Tris (pH 8.0), 1.0% NP-40 or Triton X-100, 150 mM sodium chloride, 0.1% SDS, 0.5% sodium deoxycholate, and plus protease inhibitor (Cat. No. P8340, Sigma-Aldrich, St. Louis, MO, USA). The protein concentrations in all samples were determined by a Pierce BCA Protein Assay Kit (Cat. No. 23225, Thermo Fisher Scientific).

Western Blotting

The protein levels of nuclear factor kappa B (NF-κB P65) were determined in both the cytosolic and nuclear fraction of the aorta whereas all other proteins were detected in the total cell homogenates obtained from treated cultured macrophages. Equal protein amounts (40 µg/well) were electrophoresed on 8–15% SDS–polyacrylamide gel and then electroblotted onto nitrocellulose membranes (Sigma). Membranes were then blotted overnight at 4 °C against primary polyclonal and monoclonal antibodies against p-NF-κB P65 (Ser536) (Cat. NO. sc-136548, 65 kDa, 1:500); NF-κB P65 (Cat. No. sc-8008, 65 kDa, 1:500); p-IκBα (Ser32) (Cat.

NO. sc-8404, 35 kDa, 1:1000), IκBα (Cat. No. sc-1643, 35 kDa, 1:1000), IL-1β (Cat. NO. sc-32294, 17/31 kDa, 1:1000), CD36 (Cat. No. sc-7309, 88 kDa, 1:1000), and LOX-1 (Cat. No. sc-66155, 32, 1:250), which all were supplied by Santa Cruz Biotechnology, and antibodies against p-IKKα/β (Ser176/180) (Cat. No. 2697, 85/87 kDa, 1:500), NLRP-3 (Cat. No. 15101, 110 kDa, 1:250), procaspase/caspase-1(Cat. No. 58036, 45/17, kDa 1:100), and β-actin (Cat. No. 49 67, 45 kDa, 1:3000), which all were obtained from Cell Signaling Technology. Membranes were then washed with TBS-T buffer overnight at 4 °C and then incubated with the corresponding HRP-conjugated secondary antibody. Antigen–antibody reactive reactions were detected using a Pierce ECL kit (Thermo Fisher, USA, Piscataway, NJ) and photographed and analyzed by C-DiGit Blot Scanner (LI-COR, USA). Each membrane was stripped up to four times in which the detection of the phosphorylated form was performed first, and β-actin was performed last. Data were analyzed for $n = 6$/group or treatment. Band density was normalized using the internal control, β-actin. Data were analyzed for 6 samples/group.

Histopathological Evaluation

For light microscopy, freshly frozen aortas were processed for routine Sudan black stain according to the procedure established by Sheehan and Hrapchak [47].

Statistical Analysis

Graphing and comparison between the groups were analyzed by one-way ANOVA test using GraphPad Prism (Version 6) followed by Tukey's post hoc test and the data were expressed as mean ± SD. A value of $p < 0.05$ was considered to be statistically significant.

Results

Co- or Post-*C. aronia* Treatment Lowers Circulatory Levels of LDL-c and ox-LDL-c and Enhances HDL-c only in HFD-Fed Rats

HFD-fed rats showed a significant decrease in serum levels of HDL-c with parallel increases in serum levels of LDL, ox-LDL-c and MDA (Fig. 1a–d) and had higher level of ROS in their aorta homogenate (Fig. 1e), all of which were significantly reversed in HFD-fed rats co- or post-treated with *C. aronia* with a more profound effect when the extract was co-administered (Fig. 1a–e). Interestingly, control rats + *C. aronia* had lower serum levels of MDA, LDL, and ox-LDL-c with stable serum levels of HDL-c and aortic ROS levels as compared to controls (Fig. 1a–d). Given the significant decrease in LDL-c and ox-LDL-c with co-and post-*C. aronia* therapy, these data suggest that the extract, at the tested dose, is able to decrease LDL levels which could be due to a decreased intestinal cholesterol absorption and/or conversion to LDL and/or due to their increased liver uptake. It also suggests that the extract is able to inhibit LDL-c oxidation.

Co- or Post-*C. aronia* Treatment Downregulates the Protein and mRNA Expression of Inflammatory Markers and Inhibits NF-κB P65 Nuclear Translocation in HFD-Fed Rats

LOX-1 and CD36 are major transporters of ox-LDL on the ECs and macrophages [4]. ox-LDL-c are classical regulators of LOX-1 and CD36 and major activators of the NLRP3 inflammasome and pro-IL-1β through the activation of the NF-kB [4, 48]. Once assembled and activated, the cleavage of procaspase-1 yields an active caspase-1, which converts the pro-inflammatory cytokines, IL-1β and IL-18, into their active forms to induce an inflammatory response [4]. Compared to the control, control + *C. aronia*-treated rats showed stable levels of CD36, LOX-1, NLRP-3, procaspase-1, active caspase-1, and precursor and mature forms of IL-1β (Fig. 2a–d) as well as mRNA or serum levels of TNF-α and IL-6 (Fig. 3a–d), and cytoplasmic/nuclear distribution of NF-κB P65 (Fig. 4a–c). These data suggest that *C. aronia* does not affect the expression of ox-LDL receptor and inflammasome assembly or activity under normal conditions, even in the presence of low-circulatory ox-LDL-c. All these biochemical endpoints were significantly increased with a parallel increase in aortic nuclear levels of NF-κB P65 seen in the serum or aorta of HFD-fed rats compared to control rats (Figs. 2, 3, 4), thus indicating the major role of HFD in the process of vascular inflammation which mediated, at least, via increased ox-LDL-c receptor expression and intake and subsequent activation of the NLRP-3 inflammasome and release of pro-inflammatory cytokines. However, significant decreases in the levels of all these parameters with a coincided increase in cytosolic levels of NF-κB P65 were seen in the aortas of HFD-fed rats co- or post-treated with *C. aronia* as compared to HFD-fed rats (Figs. 2, 3, 4), suggesting that these effects are most likely secondary to its hypolipidemic and antioxidant effect through the decrease in circulatory ox-LDL-c levels.

C. aronia Enhances Cell Viability, Lowers Intracellular ROS Levels, and Inhibits NF-κB Activation in Isolated Macrophages With or Without LPS Stimulation

It is well established that LPS stimulation-induced ROS can lead to an inflammatory response mediated by increasing levels of LOX-1 and CD36, activation of NF-κB, and rapid NLRP-3 inflammasome assembly [49] To investigate if the

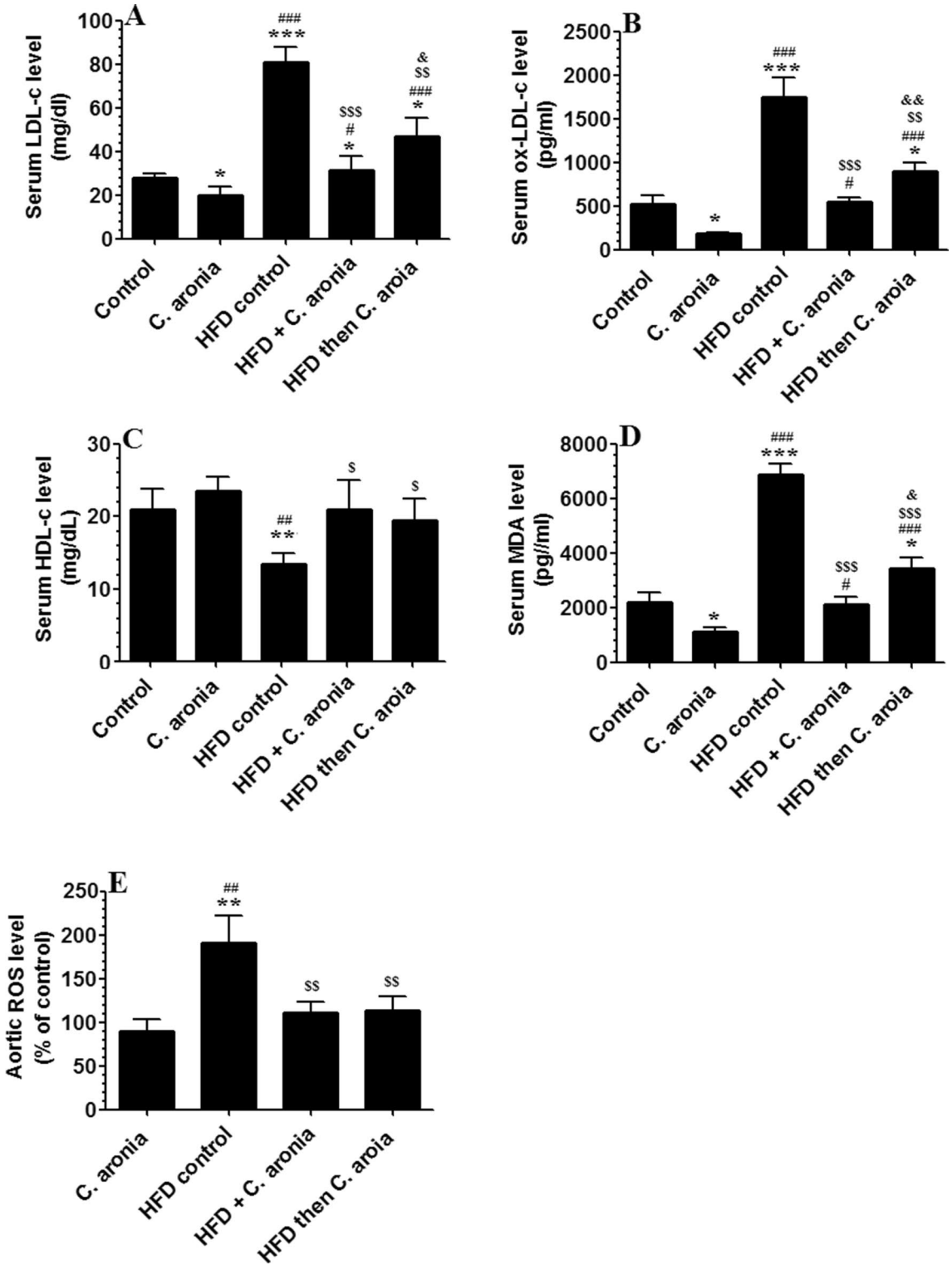

Fig. 1 Serum levels of low-density lipoprotein cholesterol (LDL-c) (**a**), oxidized low-density lipoprotein cholesterol (ox-LDL-c) (**b**), and High-density lipoprotein cholesterol (HDL-c) (**c**) and aortic levels of Malondialdehyde (MDA) (**d**) and reactive oxygen species (ROS) (**e**) in all experimental groups. Values are expressed as Mean ± SD ($n = 6$ rats/group). *,**,***: vs. Control (STD) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; #,##,###: vs. *C. aronia*-fed rats at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $,$$,$$$: vs. HFD-fed rats at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; and &,&&,&&&: vs. HFD + *C. aronia* at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

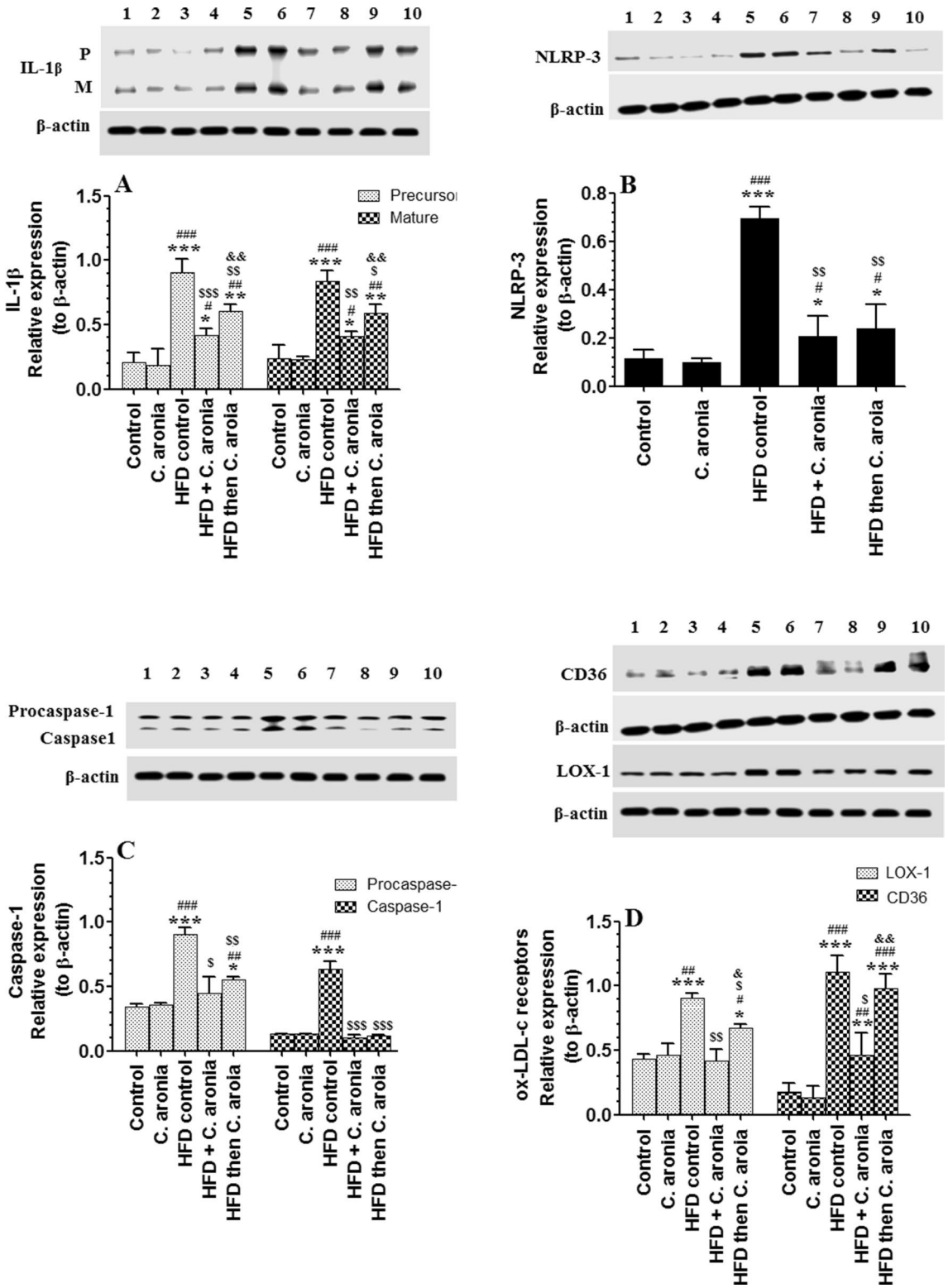

Fig. 2 Protein levels of precursor (P) and mature (M) forms of interleukin 1β (IL-1β) (**a**), NLRP-3 (**b**), caspase-1 (**c**), CD36 (**d**), and LOX-1 in the aorta of all experimental groups (**d**). Values are expressed as Mean ± SD ($n = 6$ rats/group). *,**,***: vs. Control (STD) (lanes 1 & 2) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\#,\#\#,\#\#\#}$: vs. *C. aronia*-fed rats (lanes 3 & 4) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\$,\$\$,\$\$\$}$: vs. HFD-fed rats (lanes 5 & 6) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; and $^{\&,\&\&,\&\&\&}$: vs. HFD + *C. aronia* (lanes 7 & 8) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. Lanes 9 &10 represents HFD then C. aronia-treated groups

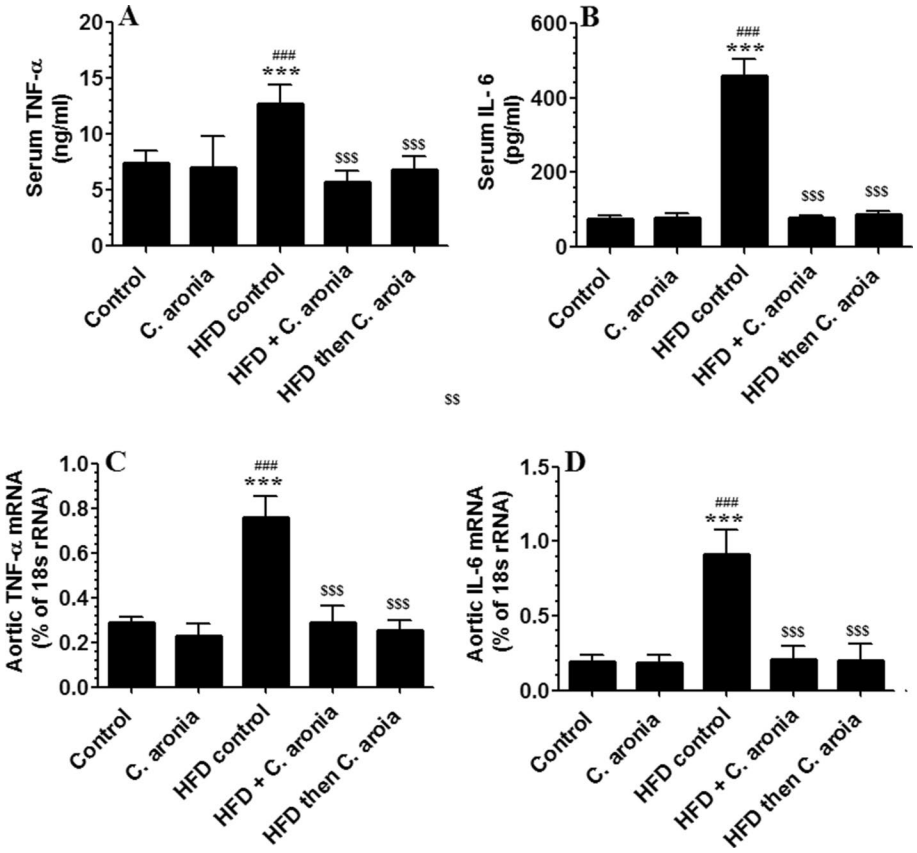

Fig. 3 Serum and aortic mRNA levels of tumor necrosis factor-α (TNF-α, **a**, **c**, respectively) and interleukin-6 (IL-6, **b**, **d**, respectively) in all experimental groups. mRNA levels of both TNF-α and IL-6 were normalized to the reference gene, 18 s rRNA. Values are expressed as Mean ± SD ($n = 6$ rats/group). ***: vs. Control (STD) at $p < 0.001$; ###: vs. *C. aronia*-fed rats at $p < 0.001$; and $$$: vs. HFD-fed rats at $p < 0.05$ $p < 0.001$, respectively; and &,&&: vs. HFD + *C. aronia* at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively

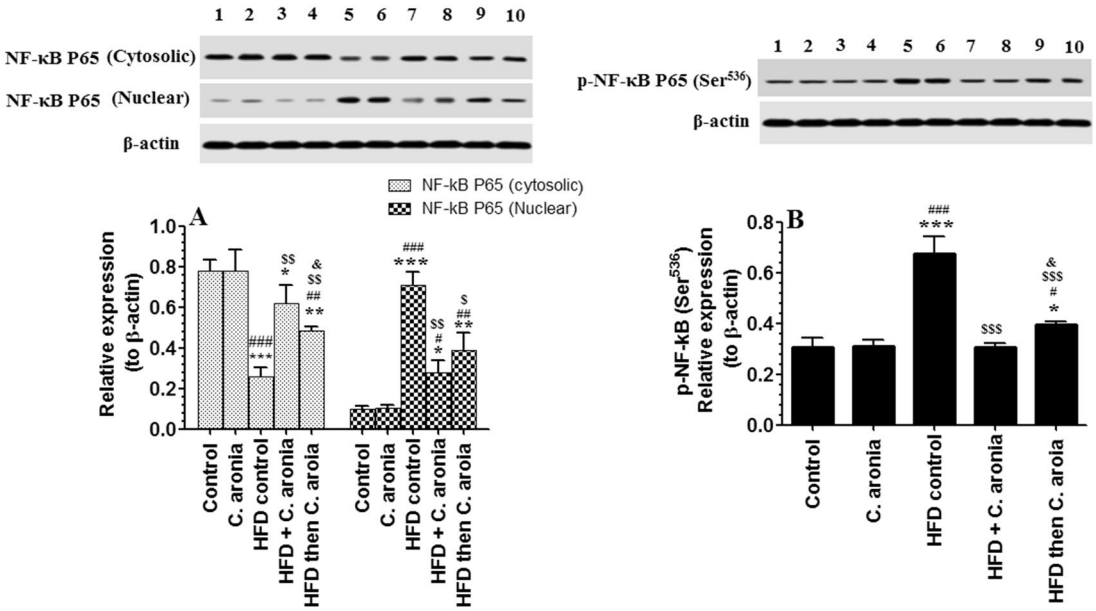

Fig. 4 Protein levels of total NF-kB P65 in the cytoplasm (**a**) and nuclear (**b**) fractions as well as and levels of p-NF-kB P65 (Ser^{536}) in all experimental groups. Values are expressed as Mean ± SD ($n = 6$ rats/group). *,**,***: vs. Control (STD) (lanes 1 & 2) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; #,##,###: vs. *C. aronia*-fed rats (lanes 3 & 4) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $,$$,$$$: vs HFD-fed rats (lanes 5 & 6) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; and &: vs. HFD + *C. aronia* (lanes 7 & 8) at $p < 0.05$. Lanes 9 &10 represent HFD then *C. aronia*-treated groups

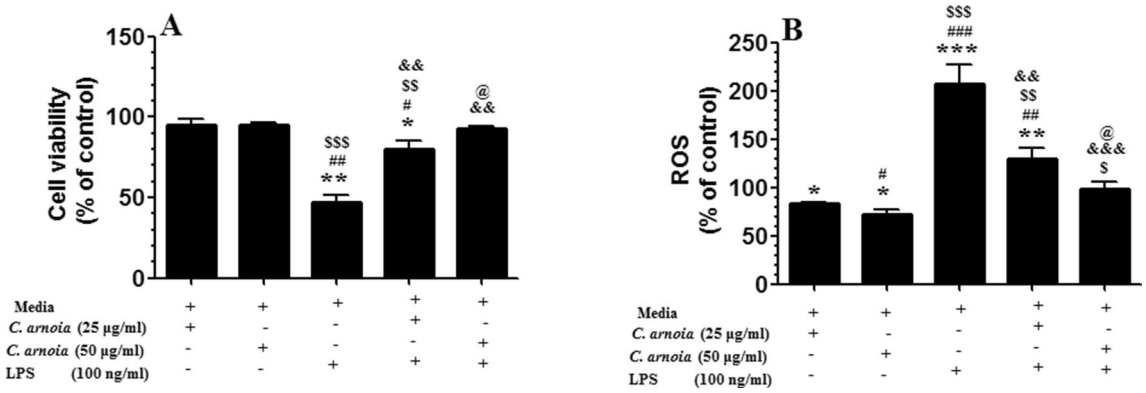

Fig. 5 Cell viability ratio and levels of reactive oxygen species (ROS) in cultured peritoneal macrophages pre-incubated with increasing concentrations of *C. aronia* (25 & 50 µ/mL) for 24 h with or without post-stimulation with lipopolysaccharides (LPS, 100 ng/mL) for the next 12 h. Values are expressed as Mean ± SD ($n = 6$ cultures/group). *·**·***: vs. Control untreated macrophages at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\#,\#\#,\#\#\#}$: vs. Macrophages pre-incubated with *C. aronia* (25 µg/mL) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\$,\$\$,\$\$\$}$: vs. Macrophages pre-incubated with *C. aronia* (50 µg/mL) $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; and $^{\&\&,\&\&\&}$: vs. Lipopolysaccharides (LPS)-stimulated macrophages at $p < 0.01$ and $p < 0.001$, respectively. $^{@}$: vs. Lipopolysaccharides (LPS) + *C. aronia* (25 µg/mL)-stimulated macrophages at $p < 0.05$

anti-inflammatory effect of *C. aronia* is independent of its hypolipidemic effect and decreasing levels of ox-LDL-c, we tested the direct effect of the extract on LPS-unstimulated or stimulated isolated macrophages (in absence of ox-LDL-c). As expected, cell viability was significantly decreased and levels of intracellular ROS were significantly increased in homogenates of LPS-stimulated macrophages (Fig. 5a, b).

Phosphorylation of NF-κB P65 and its nuclear translocation depends mainly on the stability of NF-κB-associated inhibitory protein, IkBα [49]. The latter is degraded by phosphorylation at its Ser^{32} by the activation of IKKα/β (by Ser phosphorylation at different sites) [50]. In addition, total cell homogenate levels of p-NF-κB 65(Ser^{536}), p-IkBα (Ser^{32}), and p-IKKα/β ($Ser^{176/180}$), as well as the nuclear level of NF-κB P65 were significantly increased, whereas the cytosolic level of NF-κB P65 and total levels IkBα were significantly decreased in cell homogenates of LPS-stimulated macrophages, as compared to unstimulated macrophages (Fig. 6a–d). On the other hand, in a dose-dependent manner and in both unstimulated and LPS-stimulated macrophages, *C. aronia,* at a final concentrations of 25 or 50 µg/mL, significantly increased the cytosolic levels of NF-κB P65 and total levels of IkBα, while it significantly decreased total levels of p-NF-κB 65 (Ser^{536}), p-IkBα (Ser^{32}), and p-IKKα/β ($Ser^{176/180}$). Moreover, the nuclear accumulation of NF-κB P65 and intracellular ROS levels were also significantly decreased (Figs. 5b, 6a–d). Of note, *C. aronia* at both doses did not affect cell viability of unstimulated cells but significantly increased cell viability of LPS-stimulated cells.

C. aronia Does Not Affect LOX-1 and CD36 But Downregulates NLRP-3 and IL-1β in Unstimulated Macrophages and Inhibits Downregulation of These Proteins in LPS-Stimulated Cells

Protein levels of LOX-1 and CD36 were not significantly changed in cultured macrophages, pre-incubated with *C. aronia* at both tested doses (Fig. 7a, b), while NLRP-3 and IL-1β protein levels were significantly decreased in a dose-dependent manner (Fig. 7c, d). On the other hand, levels of LOX-1, CD36, NLRP-3, and IL-1β were significantly increased in LPS-stimulated macrophages as compared to untreated cells (Fig. 7a–d). Pre-incubating LPS-stimulated macrophages with *C. aronia* at both doses significantly decreased the protein levels of all of the above-mentioned proteins as compared to untreated control cells, with the highest effect induced by the 50 µg/mL dose of *C. aronia* (Fig. 7a–d).

C. aronia Inhibits Fat Accumulation in the Aorta of Treated Rats

Histological staining techniques with Sudan Black and oil red O remain a gold standard to evaluate atherosclerosis in histological sections of ex vivo tissues [51–54]. In this study, we evaluated the accumulation of fat droplets in the aorta of all groups of rats by Sudan Black B staining (Fig. 8). The aorta of control rats co-treated with *C. aronia* showed absence of fat droplets in their tunica intima (TA), muscularis (TM), and adventitia (TA) and the thickness of these layers were similar to those observed in the control rats (Fig. 8a, b, respectively). The aortas obtained

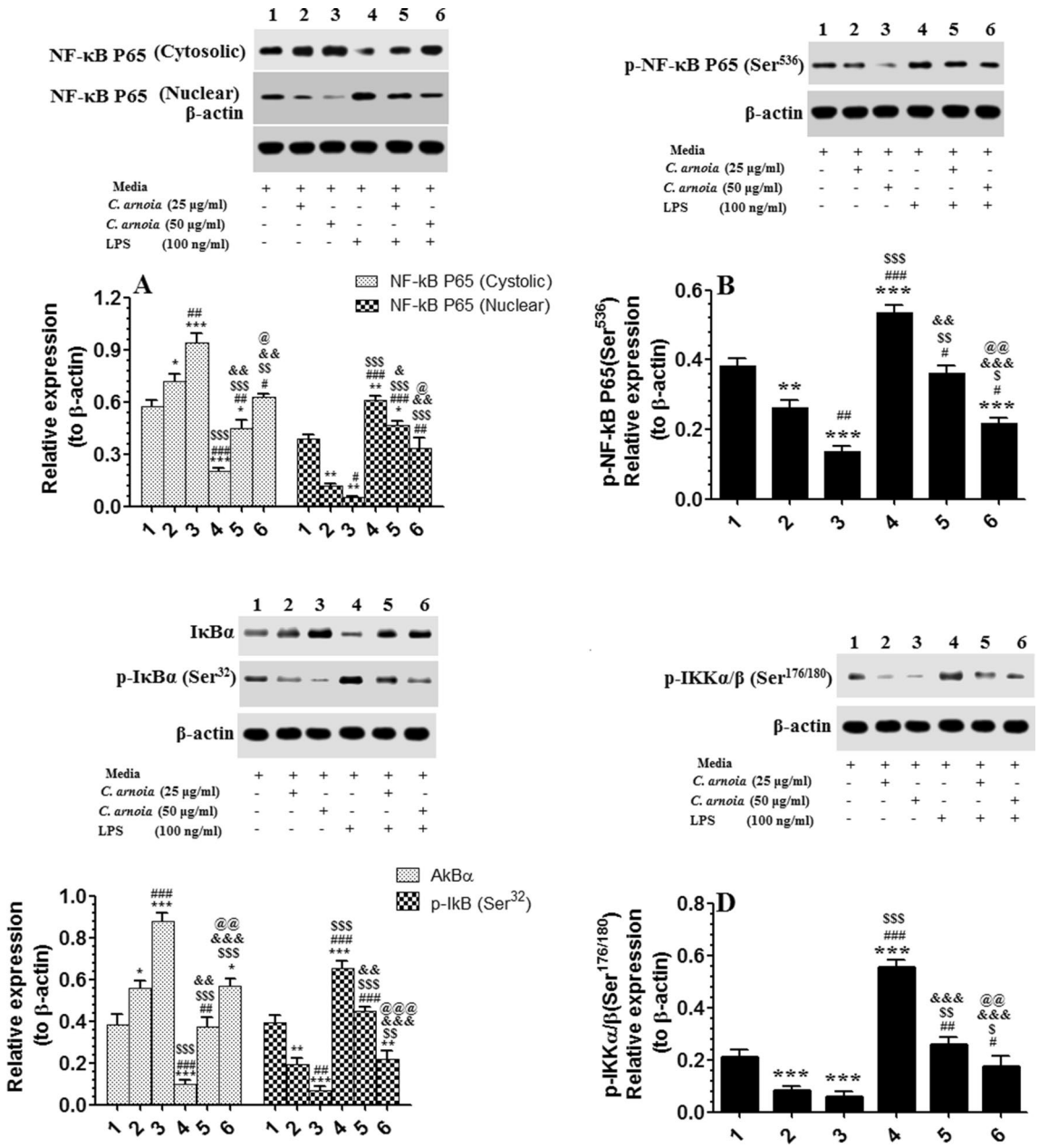

Fig. 6 Systolic and nuclear levels of NF-κB P65 (**a**) and cytoplasmic levels of phosphor-NF-κB P65 (Ser^{536}) (**b**), IkBα, phospho-IkBα (Ser^{32}) (**c**), and phospho-IKKα/β ($Ser^{176/180}$) (**d**) in the cultured peritoneal macrophages. Macrophages were pre-incubated with increasing concentrations of *C. aronia* (25&50 µ/mL) for 24 h with (lanes 4 & 5, respectively) or without (lanes 1 & 2, respectively) post-stimulation with lipopolysaccharides (LPS, 100 ng/mL) for the next 12 h. Values are expressed as Mean ± SD ($n = 6$ cultures/group). *·**·***: vs. Control untreated macrophages (lane 1) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\#,\#\#,\#\#\#}$Macrophages pre-incubated with *C. aronia* (25 µg/mL) (lane 2) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; $^{\$,\$\$,\$\$\$}$: vs. Macrophages pre-incubated with *C. aronia* (50 µg/mL) (lane 3) $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively; and $^{\&,\&\&,\&\&\&}$: vs. Lipopolysaccharides (LPS)-stimulated macrophages (lane 4) at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. $^{@,@@,@@@}$: vs. Lipopolysaccharides (LPS) + *C. aronia* (25 µg/mL)-stimulated macrophages (lane 5) at $p < 0.05$, $p < 0.01$, and $p < 0.001$. Lane 6: lipopolysaccharides (LPS) + *C. aronia* (50 µg/mL)-stimulated macrophages

from HFD-fed rats showed significant increases in fat deposition in their TM and TA with increased thickness of both layers as compared to control rats fed LFD (Fig. 8c, d). On the contrary, aortas obtained from HFD-fed rats co- or post-treated with *C. aronia* showed less deposition of fat in their TM and TA with normal thickness of their TM (Fig. 8e, f).

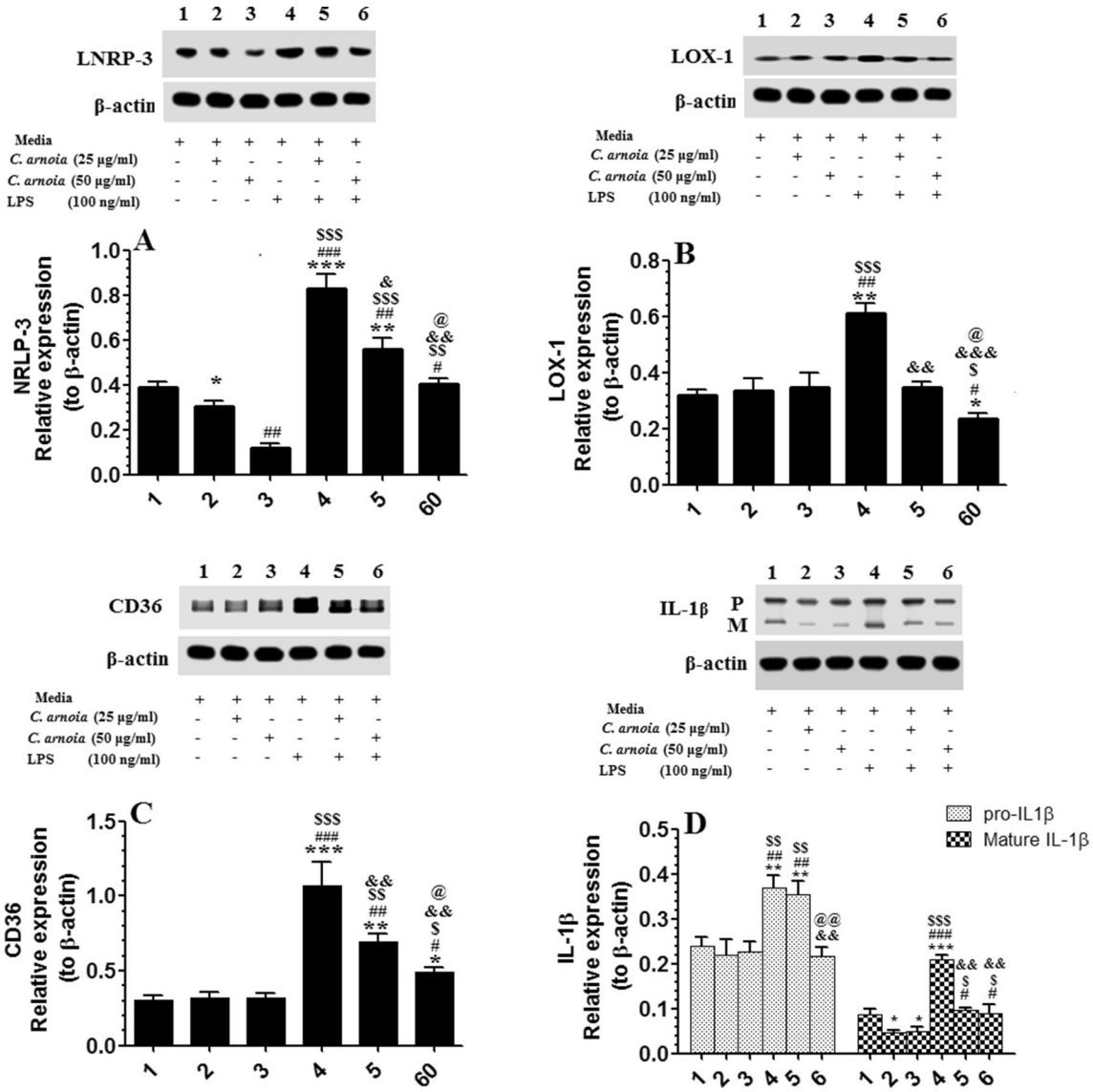

Fig. 7 Protein levels of NLRP-3 (**a**), LOX-1 (**b**), CD36 (**c**) and precursor (P) and mature (M) form of interleukin-1β (IL-1β) (**d**) in total cell homogenates of cultured peritoneal macrophages. Macrophages were pre-incubated with increasing concentrations of *C. aronia* (25&50 µ/mL) for 24 h with (lanes 4 & 5, respectively) or without (lanes 1 & 2, respectively) post-stimulation with lipopolysaccharides (LPS, 100 ng/mL) for the next 12 h. Control group received no treatment (lane 1). Values are expressed as Mean ± SD ($n=6$ cultures/group). *·**·***: vs. Control untreated macrophages (lane 1) at $p<0.05$, $p<0.01$, and $p<0.001$, respectively; $^{\#,\#\#,\#\#\#}$: vs. Macrophages pre-incubated with *C. aronia* (25 µg/mL) (lane 2) at $p<0.05$, $p<0.01$, and $p<0.001$, respectively; $^{\$,\$\$,\$\$\$}$: vs. Macrophages pre-incubated with *C. aronia* (50 µg/mL) (lane 3) $p<0.05$, $p<0.01$, and $p<0.001$, respectively; and $^{\&,\&\&,\&\&\&}$: vs. Lipopolysaccharides (LPS)-stimulated macrophages (lane 4) at $p<0.05$, $p<0.01$, and $p<0.001$, respectively. $^{@,@@}$vs. Lipopolysaccharides (LPS) + *C. aronia* (25 µg/mL)-stimulated macrophages (lane 5) at $p<0.05$ and $p<0.01$. Lane 6: lipopolysaccharides (LPS) + *C. aronia* (50 µg/mL)-stimulated macrophages

Discussion

Previous studies have confirmed the hypolipidemic, antioxidant, anti-inflammatory effects of the aqueous extract of *C. aronia* in various animal models, including those induced by HFD feeding [38, 39]. In addition, the role of the ox-LDL-c/NF-κB/NLRP-3 inflammasome/IL-1β signaling pathway in HFD-induced vascular inflammation is well documented in literature [4, 6–8, 49]. Although our recent findings in such an animal model suggest that *C. aronia* reduced the thickness of aorta and inhibited the aortic protein levels of IL-6 and TNF-β [41], it remains unknown whether this effect is mediated by a direct anti-inflammatory effect of the extract or whether it is secondary to its hypolipidemic and antioxidant effects. In addition, the effect of *C. aronia* on the NF-κB/NLRP-3 inflammasome/IL-1β axis in endothelial or

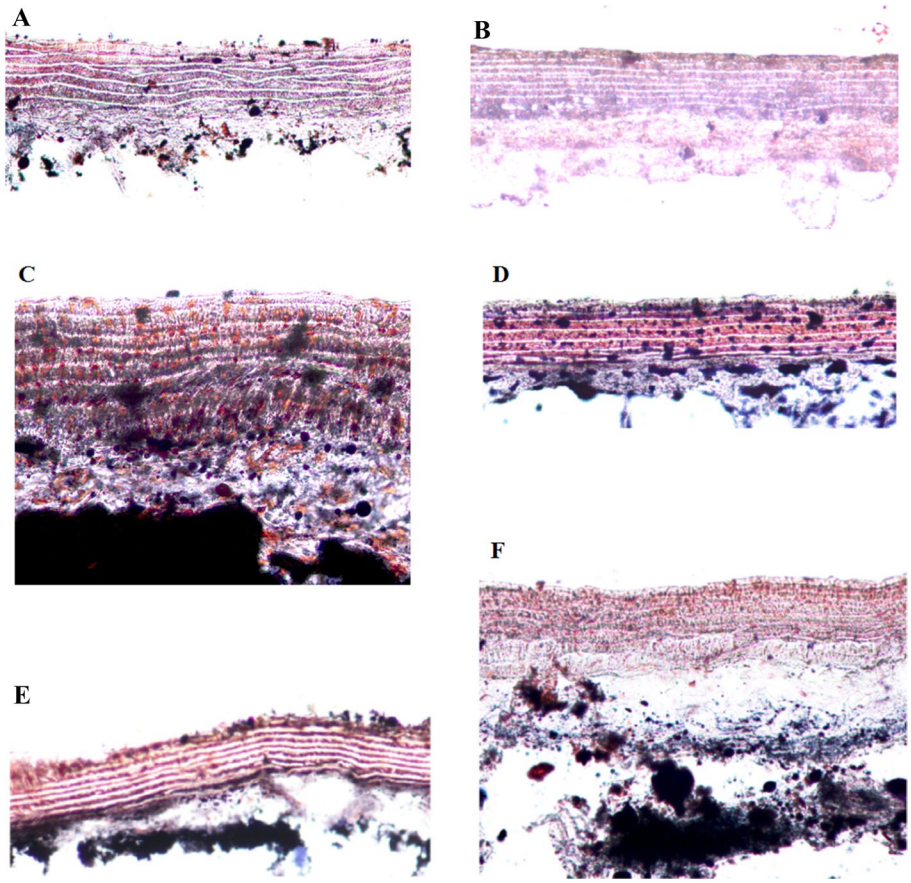

Fig. 8 Sudan Black B-stained frozen aorta sections from all experimental groups of rats. Black and dark red spots (Arrows) indicate accumulation of fat droplets. Photomicrographs **a** and **b** were taken from control rats fed the standard fat diet (STD) and received the vehicle or co-treated with *C. aronia*, respectively, with few lipid vacuoles in their tunica intima (TI) muscularis (TM) and adventitia (TA). Photomicrographs **c** and **d** were taken form high-fat diet (HFD)-fed rats and show increased accumulation of fat droplets mainly in both TM and TA. The thickness TM and TA of some areas of the aorta were increased (**c**). Photomicrographs **e** and **f** were taken from HFD co- or post-treated with *C. aronia*, respectively, and showed decrease in the thickness of TM and absence of fat droplets in this layer. However, fat accumulation appeared in the TA of both groups but much lower than observed in the TA of HFD-fed rat's aorta. 200x

macrophage cells has never been investigated before. Therefore, in this study, we investigated the effect of *C. aronia* on the aortic expression of NF-κB, the NLRP-3 inflammasome, and IL-1β in a HFD-fed rat model of induced vascular inflammation. In addition, to further test if this effect occurs independently from the decreasing levels of LDL and ox-LDL-c, we examined the expression and activity of this axis in isolated macrophages co-treated with increasing concentrations of the aqueous extract *of C. aronia* with or without LPS stimulation. Our findings showed that *C. aronia* reverses HFD-induced vascular inflammation in rats by inhibiting the NF-κB/NLRP-3 inflammasome/IL-1β axis, which is mainly due to its hypolipidemic effect, whereas in vitro it inhibits this axis in both LPS-stimulated and unstimulated macrophages accompanied with a reduction in ROS levels. These data suggest that the anti-inflammatory effect of the aqueous extract of *C. aronia* in rats is mediated by a combination of its hypolipidemic and antioxidant potential. A detailed mechanism of action is discussed below and is shown in the attached graphical abstract.

In this study, co- or post-administration of *C. aronia* to HFD-fed rats significantly lowered serum levels of MDA, LDL-c, and ox-LDL-c, increased serum levels of HDL-c and significantly inhibited aortic levels of ROS. At first glance, this could be explained by the hypolipidemic and antioxidant potential of the extract, as previously reported by us and others [38–40]. However, it was previously shown that HDL-c can inhibit the oxidation of LDL-c by its direct antioxidant activity and prevent the accumulation of lipid hydroperoxides in LDL-c particles [55]. Given the lowered levels ox-LDL-c in the sera of control rats with stable HDL-c serum and aortic ROS levels, the current findings of this study strongly suggest that the decrease in circulatory levels of ox-LDL in the sera of *C. aronia*-treated control or HFD-fed rats is most likely secondary to the subsequent decrease in the circulatory levels of LDL-c. Such hypolipidemic effect of *C. aronia* can be deduced from other similar studies which showed that various species of Hawthorn can inhibit serum levels of LDL-c by inhibiting intestinal CHOL absorption, decreasing conversion to LDL-c, and/or increased serum clearance of LDL-c via increasing the hepatic expression of LDL-c receptors [29, 30, 56].

On the other hand, the vascular uptake of ox-LDL-c by the ECs and resident/or infiltrating macrophages is mediated by LOX-1 and CD36 receptors and is associated with an increase in ROS generation from these macrophages via activation of NADPH oxidase and metalloproteinases (MMPs) [1, 4, 57]. It was shown that ox-LDL-c can upregulate the expression of LOX-1 and CD36 to increase its own uptake, thereby mediating other cellular manifestations of vascular

inflammation, including ECs dysfunction, adhesive molecule expression, and cytokine release, through the activation of the NLRP-3 inflammasome and the subsequent activation of IL-1β [4, 56]. Indeed, ox-LDL-c can cause a rapid assembly of the NLPR-3 inflammasome and induce nuclear translocation of NF-κB, in a ROS-dependent mechanism [4, 58].

In accordance and associated with the higher serum levels of ox-LDL-c, the aortas of HFD-fed rats show significantly higher protein levels of LOX-1 and CD36 with a parallel increase in total ROS in their aortic homogenates which were completely reversed by post- or co-treatment of *C. aronia*. Such an increase in ROS could be attributed to increased ox-LDL uptake by the macrophages, as explained above. These data are in accordance with the above-mentioned studies and confirm the pro-oxidant roles of the ox-LDL-c/LOX-1/CD36 signaling pathway. Of note, the levels of ROS and expression levels of LOX-1 and CD36 were not significantly changed in the aorta of control rats. These data demonstrate the *C. aronia* has no regulatory effect on the expression of LOX-1 and CD36, and such decrease in their expression in the aortas of *C. aronia* co- or post-treated HFD-fed rats is secondary to the lower circulatory levels ox-LDL-c and their reduced uptake. It also suggests that only increased circulatory ox-LDL-c levels could enhance the expression of both LOX-1/CD36, as stable expression levels of both receptors were seen in control rats co-treated with the extract, even in the presence of reduced ox-LDL-c levels.

In addition, the vascular anti-inflammatory effect of *C. aronia* in this animal model was also confirmed by the lower nuclear levels of NF-κB and subsequent decrease in the protein levels of all components of the NLPR-3 inflammasome, including NLPR-3, caspase-1, and mature IL-1β, as well as by the significant decrease in TNF-α and IL-6 mRNA levels. This is in accordance with our previous findings that showed lower TNF-α and IL-6 protein levels in the aorta of HFD-fed rats that were co or post-treated with *C. aronia* [41]. Interestingly, *C. aronia* did not affect the expression of any of these inflammatory markers in control rats. These data are in line with the above-mentioned findings which suggest that such inhibition of NF-κB and the NLPR-3 inflammasome is mediated by lowering the circulatory levels of ox-LDL-c particles and their uptake by ECs and macrophages. In addition, it has been shown that HDL-c inhibits CHOL crystal-induced inflammation and subsequent activation of the NLRP-3 inflammasome in THP1 cells and in monocyte-derived macrophages by its direct inhibition of NF-κB signaling and its direct binding on CHOL crystals [59]. Hence, given the higher levels of HDL-c in the sera of HFD-fed rats, which were co- or post-treated with *C. aronia*, this extract's anti-inflammatory effect could be related to its role in increasing and restoring the HDL-c levels in these treated HFD-fed rats.

In spite of these findings, which clearly suggest that the in vivo anti-inflammatory effect of *C. aronia* is mainly mediated by its hypolipidemic effect and the decrease in aorta ROS levels is secondary to the inhibition of ox-LDL-c uptake by the macrophages, our data cannot definitely exclude that the extract's antioxidant activity plays a role in its anti-inflammatory effect. Unfortunately, we could not examine this in our in vivo model, but deserved further attention given the upregulatory role of ROS on LOX-1 and CD36 and their stimulatory role on NF-κB activity and subsequent activation of the NLPR-3 inflammasome [4]. Indeed, several studies have suggested that *C. aronia* has an antioxidant potential in vivo and in vitro. For example, the aqueous extract of *C. aronia* protected the testis and preserved sperm quantity and quality, by upregulation of the nuclear 2-related factor 2 (Nrf2)-induced SOD and glutathione (GSH) [39]. Similarly, other studies have shown that *C. aronia* displays ROS-scavenging activity and is able to prevent Fe^{2+}-induced lipid peroxidation in rat liver homogenates [35].

Hence, we further examined if the antioxidant potential could also mediate the anti-inflammatory effect of *C. aronia* in vitro in LPS-stimulated isolated macrophages. We selected this model as LPS stimulation of cultured macrophages results in a rapid increase in ROS and increases LOX-1 and CD36 levels [49]. In addition, LPS induced ROS rapidly and directly activates NF-κB and the NLRP-3 inflammasome [49]. Hence, this model allows us to study the anti-inflammatory effect of the extract in the absence of ox-LDL-c to exclude its effect and provides direct evidence of its antioxidant potential.

In this part of the study, *C. aronia*, in a dose-dependent manner, inhibited ROS in LPS-stimulated and unstimulated macrophages. It also inhibited protein levels of LOX-1 and CD36 but only in LPS-stimulated cells, thus demonstrating the absence of a regulatory effect of the extract on the expression of these receptors. These data support the in vivo evidence and suggest that the decreased expression of these receptors is mediated either by the decreased ox-LDL-c levels or due to inhibition of ROS generation. In addition, the extract in both LPS-stimulated and unstimulated cells resulted in a dose-dependent decrease in the expression of NLPR-3 and the mature form of IL-1β, with a parallel significant decrease in the nuclear levels of NF-κB P65 and total levels of p-NF-KB P65, p-Ikβα, and p-IKK, and a significant increase in total cell levels of Ikβα, indicating that *C. aronia* inhibits NF-κB/NLRP-3 inflammasome priming. Again these effects were associated with reduced levels of ROS, independent of ox-LDL-c and HDL-c levels. On the basis of these data, the in vitro part of this study supported the in vivo study and suggested that the extract could also inhibit HFD-induced vascular inflammation in rats by inhibiting or scavenging ROS generation. However, if this anti-inflammatory is ROS independent, it cannot be concluded from this

study and future studies, using a ROS-scavenging agent in combination with the extract, are needed to elucidate this. Similar to the findings of this study, leaf extracts prepared from *C. pinnatifida* attenuated atherosclerosis development in apoE knock-out mice by lowering LDL-c and increasing SOD1 and SOD2 mRNA expression [34].

However, why the extract inhibited ROS, expression of the NLRP-3 inflammasome, and activity of NF-κB in control unstimulated macrophages, but failed to induce similar effects in the aorta of control rats need further clarification. At this stage, we cannot draw further conclusions about the variation in these results. However, it could be possible that the extract exerts different effects depending on the cell type, with the main cell types present in vivo in the aorta being endothelium and smooth muscles cells. As ROS is mainly induced in the macrophages by NADPH oxidase, it could be possible that the extract inhibits this enzyme in the isolated macrophages. Also, the only variable between the two models is the stable levels of HDL-c levels in the serum of treated rats co-treated with *C. aronia*. As mentioned before, HDL-c can regulate NF-κB activity and hence the NLRP-3 inflammasome assembly [59]. Therefore, it seems that normal levels of HDL-c in the rat sera prevented the inhibitory effect of the extract on NF-κB activity. However, this hypothesis needs further investigation.

In spite of these interesting findings, this study still has some limitations. Most important is that the active ingredients of the aqueous extract of *C. aronia,* responsible for the observed hypolipidemic, antioxidant, and anti-inflammatory effects, were not determined. In fact, we have previously examined the phytochemical composition of the aqueous extract of *C. aronia,* which showed the presence of polyphenols including flavonoids, terpenoids, and organic acids [36]. Similar to this screening, several other studies have screened the chemical composition of various Hawthorn species including *C. monogyna, C. azarolus, C. pinnatifida*, and *C. oxyacantha,* revealing similar results to ours [13–17, 29, 60–63]. Interestingly, the chemical composition of Hawthorn species was almost identical between these different species and polyphenols were abundant in the leaves, fruits, and flowers of the plant [13–17, 29, 60–63].

In this regard, it was shown that the most prominent flavonoid constituents in these different Hawthorn species include quercetin, quercitrin, catechin, rutin and vitexin, vitexin-2′′-O-rhamnoside, vitexin-4′-acetyl-2′-rhamnoside, and oligomeric proanthocyanidin, whereas the common terpenoids include oleanolic acid, chlorogenic acid, ursolic acid, and crataegus acid [13–17, 29, 60–63]. In addition, some other species contained an abundant amount of tocopherols, ascorbic acid, and showed a good n-6/n-3 fatty acid ratio [15].

In multiple studies, it was shown that several components, such as vitexin-2″-O-rhamnoside, catechin, B2 procyanidin, quercetin, quercitrin, chlorogenic acid, and triterpenic acids, possess hypolipidemic effects via several mechanisms including the lowering of total CHOL and LDL-c levels, increasing the number of hepatic LDL receptors, preventing oxidation of LDL-c, and inhibiting intestinal ACAT activity [33, 64–67]. Additionally, other components such as flavones, including catechins, and proanthocyanidins, tocopherols, and ascorbic acid are potent antioxidants [15, 45, 68]. Hence, further studies are needed to determine which active ingredients are present in the fruit, leaves, and flowers of *C. aronia* and to identify which of these are responsible for the effects observed in this study.

Overall, these data could validate *C. aronia* as an effective medicinal plant to treat hyperlipidemia-induced vascular inflammation via inhibition of IL-1β release, mediated by direct inhibition of NF-κB-induced priming of the NLRP-3 inflammasome. Although the antioxidant potential of the extract could play a role in vitro, it is most likely that the anti-inflammatory effect of the extract is due to its hypolipidemic effect mediated by decreasing circulatory LDL-c and ox-LDL-c.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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