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Research Article

Comparative Effects of Olive Oil Polyphenols and Atorvastatin on Hypercholesterolemia-Induced Biochemical and Histopathological Changes in Rats Spleen and Lymph Nodes

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Abstract | Hyperlipidemia is a major global health concern associated with cardiovascular and metabolic disorders. Although the antilipid effects of olive oil have been widely studied, the specific roles of its bioactive compounds, particularly polyphenols such as hydroxytyrosol, require further investigation. This study aimed to evaluate the therapeutic potential of olive oil and hydroxytyrosol in comparison with atorvastatin, a conventional hypolipidemic drug, in white male Sprague Dawley rats with cholesterol-induced hyperlipidemia. Hyperlipidemia was induced through a cholesterol-rich diet. Serum levels of leptin, C-reactive protein (CRP), interleukin-12 (IL-12), malondialdehyde (MDA), and antioxidant enzymes were measured. Histopathological analysis of the spleen and lymph nodes was performed to assess tissue alterations, including fibrosis, cellular degeneration, and immune cell infiltration. Our findings demonstrated significant reductions ($p<0.05$) in serum leptin, CRP, IL-12 and MDA levels and significant increase ($p<0.05$) in total glutathione (GSH), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities in the treatment groups, particularly in those receiving a combination of olive oil polyphenols and atorvastatin. Additionally, marked improvements in spleen and lymph node histoarchitecture were observed. These results indicate that olive oil polyphenols, especially hydroxytyrosol, possess strong anti-hyperlipidemic, antioxidant, and histoprotective properties. Their combined administration with olive oil enhances therapeutic outcomes, supporting their potential as complementary or alternative interventions for hyperlipidemia management.

Keywords | Hyperlipidemia, Olive oil, Hydroxytyrosol, Atorvastatin, Spleen, Lymph nodes

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INTRODUCTION

Hyperlipidemia, particularly that induced by hypercholesterolemia, remains a major contributor to global morbidity and mortality due to its strong association with cardiovascular and metabolic disorders such as atherosclerosis, myocardial infarction, stroke, obesity, and pancreatic dysfunction (Wiyono *et al*., 2023). Diets high in saturated fats are a critical factor in the pathogenesis of hyperlipidemia, influencing the physiological regulation

of leptin secretion a hormone closely associated with lipid metabolism. Elevated fat intake induces leptin release, which, through hepatic mechanisms, enhances fatty acid oxidation and mitochondrial respiration. However, this process also contributes to oxidative stress, assumed leptin's role in promoting fibrogenesis (Martínez-Uña *et al*., 2020).

Leptin plays a dual role in physiological regulation. While moderate levels support normal metabolic processes, chronically elevated levels have deleterious effects across various organ systems. High circulating leptin is not only indicative of metabolic syndrome and insulin resistance in both sexes, but has also been implicated in oncogenic processes, particularly in females. Therefore, maintaining leptin within physiological limits is crucial for metabolic homeostasis (Misch and Puthanveetil, 2022).

Furthermore, leptin acts as a nutrient-sensing hormone, influencing immune function. It upregulates the expression of its receptors on T cells, thereby enhancing these cells' sensitivity to insulin and nutrient availability. Leptin signaling in T cells promotes glucose uptake, lactate production, cellular proliferation, and the synthesis of pro-inflammatory cytokines, thus playing a pivotal role in immune-metabolic interactions (De Blasio *et al*., 2018; Han *et al*., 2018).

Recent studies have highlighted the immuno-metabolic functions of the spleen and lymph nodes. These secondary lymphoid organs serve as extramedullary hematopoietic sites that contribute significantly to inflammatory responses. The spleen, in particular, houses reservoirs of myeloid progenitors and monocytes that are rapidly mobilized during inflammation. Additionally, it plays a central role in lipid metabolism and the regulation of immune cell development and function (Fernández-García *et al*., 2020).

Current strategies for managing hyperlipidemia include dietary modifications, regular physical activity, and pharmacological interventions aimed at reducing lipid levels. Atorvastatin is a widely used hypolipidemic drug (lipid-lowering agent) belonging to the statin class. It works by inhibiting 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a key enzyme in the liver responsible for cholesterol synthesis. Atorvastatin is especially effective in patients with familial hypercholesterolemia or mixed dyslipidemia, and it is often prescribed long-term for cardiovascular risk reduction. However, many synthetic lipid-lowering agents are associated with side effects and limited efficacy across different lipoprotein profiles. Drug resistance and adverse reactions have led to increased interest in plant-based therapies, particularly those rich in natural antioxidants. These compounds offer a safer alternative to synthetic drugs, given their diverse pharmacological effects and lower risk profiles (Khan *et al*., 2017; Parasuraman *et al*., 2019; Aljaff *et al*., 2023; Ahmed *et al*., 2023).

Among these, olive oil (OO) stands out as a functional food with substantial health-promoting properties. Rich in monounsaturated fatty acids and bioactive polyphenolic compounds, olive oil exhibits potent anti-inflammatory and antioxidant activities. Olive oil is rich in bioactive constituents including oleuropein, hydroxytyrosol, tyrosol, and other phenolic compounds, which contribute to its antioxidant, anti-inflammatory, and lipid-lowering properties (Covas *et al*., 2006; López-Miranda *et al*., 2010). Its components, such as hydroxytyrosol, have been extensively studied for their protective roles in cardiovascular and metabolic disorders, making it a promising candidate in the management of hyperlipidemia (Dalal *et al*., 2023). Several studies have documented the hypolipidemic and antioxidant potential of OO polyphenols. For instance, Castanea *et al*. (2011) demonstrated that daily intake of extra virgin OO enriched with polyphenols significantly reduced LDL oxidation in humans. Similarly, Rietjens *et al*. (2007) confirmed the ability of hydroxytyrosol to scavenge free radicals and modulate oxidative stress biomarkers. Although numerous studies have evaluated the cardiovascular benefits of OO and statins, there is limited comparative research on the impact of OO polyphenols, particularly hydroxytyrosol versus atorvastatin in preventing hyperlipidemia-induced biochemical and histopathological changes in immune-related organs like the spleen and lymph nodes. The present study attempted to investigate and compare the therapeutic effects of atorvastatin, OO, and its key polyphenol hydroxytyrosol on biochemical markers and histopathological alterations in the spleen and lymph nodes of male Sprague Dawley rats with cholesterol-induced hyperlipidemia.

MATERIALS AND METHODS

Materials

Olive Oil (OO) was extracted by thermal pressing method from Kirkuk city, Iraq. It was administered to rats at a dose of 1/2 ml/kg of body weight, while Biotechnology-Shaanxi Company provided the Hydroxytyrosol (HXT). It was used in rats at a concentration of 50 µl/kg, while rats were provided with atorvastatin (ATOR) at a dosage of 2.06 mg/kg of body mass (Ahmed, 2023).

Animals

Thirty white male Sprague Dawley rats, aged 4–4.5 months and weighing 230±30 g, were used in this study. The rats were housed in sterile cages under controlled laboratory conditions, with a temperature of 22±2 °C and a regulated light–dark cycle. Animals had free access to food and water for two weeks to acclimate to the laboratory environment. This study was approved by the Scientific Committee,

College of Education for Pure Science, University of Kirkuk, Ministry of Higher Education and Scientific Research (Approval Number: 00331).

Experiment design

In this investigation, thirty male Sprague Dawley rats were divided into six groups, each containing five animals with similar body weights, as follows:

- G0–Control group: Rats received standard chow throughout the 8-week study period.
- GI–Hypercholesterolemia (HC) group: Rats were fed a 2% cholesterol-enriched diet for the duration of the experiment, following the protocol of Noh *et al.* (2022).
- G II – HC + Olive Oil (OO) group: Rats were fed a cholesterol-rich diet and, from the 3rd to the 8th week, received virgin olive oil via oral gavage at 0.5 mL/kg/day, starting from the first day of cholesterol feeding (Mohagheghi *et al.*, 2010; Albrahim *et al.*, 2022).
- G III – HC + Hydroxytyrosol (HXT) group: Rats were fed a cholesterol-rich diet and, from the 3rd to the 8th week, were gavaged with HXT at 10 mg/kg/day orally, as described by Sookoian *et al.* (2011).
- G IV – HC + OO + HXT group: Rats were fed a cholesterol-rich diet and, from the 3rd to the 8th week, received both OO and HXT at the same doses and duration as in GII and GIII.
- G V – HC + Atorvastatin (ATOR) group: Rats were fed a cholesterol-rich diet and, from the 3rd to the 8th week, received atorvastatin at 10 mg/kg/day orally (Gocmen *et al.*, 2013).

To induce hypercholesterolemia, rats were initially fed a 5% cholesterol-enriched diet for 3 weeks (Gocmen *et al.*, 2013). The diet formulations were as follows: 2% cholesterol diet – 2 g cholesterol per 100 g standard chow; 5% cholesterol diet – 5 g cholesterol, 2 g cholic acid, and 93 g standard chow per 100 g diet. These formulations are consistent with previously validated hypercholesterolemic models (Xiao *et al.*, 2017; Zingg, 2007).

Blood sample collection

In the eighth week of the experiment, the animals were starved for 12 hours. Then, the rats were intramuscularly injected with xylazine and ketamine at doses of 5-35 mg/kg body weight to induce anesthesia. Blood was collected from anesthetized rats by cardiac puncture, left to clot at room temperature, and then centrifuged at 3000 rpm for 15 minutes to separate the serum. The serum samples were stored at −80°C until analysis. The spleen and lymphatic nodule (mesenteric and inguinal lymph nodes) were removed for tissue examination.

Physiochemical evaluations of serum samples

Serum leptin, interleukin-12 (IL-12), and C-reactive protein (CRP) levels were measured using commercial ELISA kits according to the manufacturers' instructions. For leptin (Rat Leptin ELISA Kit, ab100773, Abcam, UK), IL-12 (Rat IL-12 ELISA Kit, ab119539, Abcam, UK), and CRP (Rat CRP ELISA Kit, MBS2507397, MyBioSource, USA), standards and serum samples were added to antibody-coated wells, followed by a biotin-labeled detection antibody and an HRP-streptavidin conjugate. Color development was achieved using a TMB substrate, and optical density was measured at 450 nm. Concentrations of each analyte were calculated from standard curves generated from known concentrations (Zhang *et al.*, 1994; Sharma *et al.*, 2005; Kohut *et al.*, 2005).

Oxidative stress markers

Spleen tissue was weighed, rinsed with cold saline to remove blood, and homogenized in an appropriate cold buffer (e.g., phosphate-buffered saline or potassium phosphate buffer, pH 7.4) at a ratio of 1:10 (w/v) using a tissue homogenizer. The homogenate was centrifuged at 10,000 rpm for 15 minutes at 4 °C, and the resulting supernatant was collected for subsequent biochemical analyses viz below:

A. Total Glutathione (GSH, a nonenzymatic antioxidant): The measurement was carried out using a modified version of the method described by Beutler (1963). Absorbance was read at 412 nm, and the results were reported as micromoles per milligram of protein (μmol/mg protein).
B. Malondialdehyde (MDA): Lipid peroxidation levels were assessed by measuring malondialdehyde (MDA) concentrations using the method described by Draper and Hadley (1990). This method is based on the spectrophotometric detection of a colored complex formed by the reaction of MDA with thiobarbituric acid (TBA) spleen tissues were individually homogenized in 1 ml of Tris-HCl buffer (50 mM, pH 7.5), followed by centrifugation at 1000×g for 10 minutes. To 0.5 ml of the homogenate, 2.5 ml of trichloroacetic acid (TCA) was added, and the mixture was incubated in a boiling water bath for 15 minutes. After cooling under running tap water, the samples were centrifuged again at 1000×g for 10 minutes. Then, 2 ml of the resulting supernatant was mixed with 1 ml of TBA solution and heated in a boiling water bath for another 15 minutes. After cooling, absorbance was read at 532 nm. MDA levels were expressed in μmol/mg of protein.
C. Superoxide Dismutase (SOD – Antioxidant Enzyme): SOD activity was evaluated using the pyrogallol autoxidation method as described by Woolliams *et al.* (1983). Absorbance was measured at 420 nm, with changes in optical density monitored over a 3-minute period. The assay relies on the enzyme's ability to inhibit pyrogallol autoxidation. For the procedure, 50

µl of tissue homogenate was added to 2.85 ml of Tris-succinate buffer (0.05 M, pH 8.2), and the reaction was initiated by adding 100 µl of 8.0 mM pyrogallol. Absorbance was recorded every 30 seconds over 3 minutes at 420 nm. A control reaction containing distilled water instead of homogenate was used as a blank. SOD activity was reported as IU/mg protein.

D. Glutathione Peroxidase (GSHPx–Antioxidant Enzyme): GSHPx activity was determined following the method of Woolliams *et al*. (1983). The assay measured the rate of NADPH oxidation at 340 nm in the presence of hydrogen peroxide (H_2O_2), reduced glutathione (GSH), and glutathione reductase. Enzyme activity was calculated based on the decrease in absorbance, and results were expressed as IU/mg protein.

Histopathological study

Tissues from the animals' spleens and lymph nodes were fixed in 10% formalin saline and processed to prepare 5-µm-thick paraffin sections for hematoxylin and eosin (H and E) stain for assessment of histopathological changes (Bancroft and Gamble, 2008).

Histo-morphometric study

Sections stained with Hematoxylin and eosin (H and E) were morphometrically analyzed using Leica Qwin500 Image Analyzer Computer System (England). Assessment of the histological changes in the spleens and lymph nodes of the treated animals was achieved through the scoring for evaluation of the damage, described by Baybutt *et al*. (2002). Sections were examined for the presence of rupture, nucleolar hypertrophy, hemolysis, congestions, hemorrhage, inflammatory infiltration, macrophage, karyolysis, necrosis degeneration, fibrosis in spleen-stained sections. increased macrophage, lymphocyte infiltration, pulp expansion, lymphocyte degeneration, lymphatic nodule destruction, and congestion for lymph nodes-stained sections. All the parameters were measured for the randomly chosen five fields per section in total five sections from 5 rats in each group and reported as mean ± standard deviation.

Statistical analysis

Data for all groups were expressed as mean ± standard deviation (X±SD). SPSS program (Statistical Package for Social Science) version 16.0 (SPSS Inc., 2007) was used. Statistically, a significant difference was determined by one-way analysis of variance (ANOVA), followed by Tukey test for multiple comparisons between different groups. The test results were considered significant at $p < 0.05$, highly significant at $p < 0.01$, and very highly significant at $p < 0.001$.

RESULTS

Concentrations of Leptin, CRP and IL-12

The hypercholesterolemic group (G1) showed significant elevations in serum levels of leptin, CRP, and IL-12 compared to the control. Notably, all treatment groups (GII–GV) showed a significant reduction in these markers, with the combination of olive oil and hydroxytyrosol (GIV) being highly significant reduction. Atorvastatin (GV) also significantly reduced these markers (Table 1).

Tissue oxidative stress markers

Table 2 presents the detailed levels of the oxidative stress marker MDA and antioxidant markers GSH, SOD, and Px in the studied groups. Hypercholesterolemic rats (G1) exhibited significant reduced antioxidant enzyme levels and significantly elevated MDA. Treatments with olive oil (GII), hydroxytyrosol (GIII), their combination (GIV), and atorvastatin (GV) significantly ameliorated this imbalance. The GIV group displayed a highly significant improvement with a particular favorable profile, with higher antioxidant activity and lower MDA levels, nearly approaching control levels.

Table 1: Effect of olive oil active compounds and atorvastatin on serum levels of leptin, CRP, and IL-12 in the studied groups.

IL-12 (pg/ml)	CRP (mg/dl)	Leptin (ng/ml)	Parameters/Groups
77.800±3.962	0.953±0.0161	0.975±0.025	Control (G0)
245.000±8.631a	7.510±0.364a	8.639±0.264a	Hypercholesterolemia group (G1)
92.000±10.368*b	2.731±0.218*b	2.649±0.304*b	Hypercholesterolemia and olive oil group (GII)
91.600±5.941*b^	2.577±0.145*b^	2.609±0.264*b^	Hypercholesterolemia and Hydroxytyrosol group (GIII)
81.200±5.263#bcd	1.682±0.211#bcd	1.711±0.232#bcd	Hypercholesterolemia and (olive oil and hydroxytyrosol group (GIV)
99.000±9.823abcde	2.430±0.203abcde	2.246±0.197abcde	Hypercholesterolemia and Atorvastatin groups (GV)
348.7	561.1	721.4	F
<0.001**	<0.001**	<0.001**	p-value

a: statistically highly significant (p<0.001), *: statistically significant (p<0.05), and #: statistically non-significant (p>0.05) compared to control group, b: statistically highly significant (p<0.001) compared to Hypercholesterolemia group (G1), ^: statistically non-significant (p>0.05) and c: statistically highly significant (p<0.001) compared to Hypercholesterolemia and olive oil group (GII), d: statistically highly significant (p<0.001) compared to Hypercholesterolemia and Hydroxytyrosol group (GIII), e: statistically highly significant (p<0.001) compared to Hypercholesterolemia and (Olive Oil and Hydroxytyrosol) group (GIV).

Table 2: Oxidative stress marker (MDA) and antioxidant markers (GSH, SOD, GPx) in the studied groups.

Groups	Control (G0)	Hyper-cholesterolemia group (G1)	Hypercho-lesterolemia and olive oil group (GII)	Hypercho-lesterolemia and Hydroxy-tyrosol group (GIII)	Hypercholes-terolemia and (Olive Oil and Hydroxytyrosol group (GIV)	Hypercholes-terolemia and Atorvastatin groups (GV)	F	P-value
Total glutathione (GSH) µmol/mg proteins	4.91 ± 0.17	0.61 ± 0.02a	2.24 ± 0.06*b	2.55± 0.75*b^	3.32 ± 0.04#bcd	2.81± 0.16ab-cde	61.13	<0.001**
Malondialdehyde (MDA) µmol/mg proteins	1.66 ± 0.40	13.69 ± 1.43a	3.38 ± 1.33*b	2.66 ± 0.91*b^	2.17± 1.20#bcd	4.00± 0.51ab-cde	95.00	<0.001**
Superoxide dismutase (SOD) IU/mg protein	74.50 ± 4.32	26.64 ± 3.91a	51.66 ± 4.36*b	52.50 ± 2.8*b^	60.51 ± 1.9#bcd	55.70 ± 1.92abcde	107.6	<0.001**
Glutathione peroxidase IU/mg protein	280.66 ± 8.21	116.5 ± 7.09a	259.3 ± 1.06*b	258.16 ± 8.0*b^	271.66 ± 5.31#bcd	265.3 ± 0.07abcde	24.04	<0.001**

a: statistically highly significant (p<0.001), *: statistically significant (p<0.05), and #: statistically non-significant (p>0.05) compared to control group, b: statistically highly significant (p<0.001) compared to Hypercholesterolemia group (G1), ^: statistically non-significant (p>0.05) and c: statistically highly significant (p<0.001) compared to Hypercholesterolemia and olive oil group (GII), d: statistically highly significant (p<0.001) compared to Hypercholesterolemia and Hydroxytyrosol group (GIII), e: statistically highly significant (p<0.001) compared to Hypercholesterolemia and (Olive Oil and Hydroxytyrosol) group (GIV). GSH, SOD and GPx.

Histological analysis of the spleen

In the control group, spleen histology appeared normal, displaying clear structural features such as the trabeculae (T), red pulp (RP), and white pulp (WP), as illustrated in Figure 1a. In contrast, rats in the Hypercholesterolemic group (HC) exhibited extensive histopathological abnormalities, including fibrosis (Fi), degeneration (D), macrophage infiltration (M), inflammatory infiltration (II), hemorrhage (H), congestion (CON), hemolysis (H), nucleolar hypertrophy (NH), and marked rupture (R), as well as moderate karyolysis (KL) and necrosis (N), as shown in Figure 1b-d.

Treatment with olive oil alone in the Hypercholesterolemic group (HC) led to partial recovery, with fibrosis, degeneration, macrophage presence, and inflammatory infiltration reduced to a moderate degree, and other abnormalities such as necrosis, karyolysis, hemorrhage, NH, and rupture reduced to be mild or trace levels (Figure 1e). The HXT-only group showed slightly better improvement, with most lesions, including fibrosis, hemorrhage, NH, rupture, and inflammatory infiltration, reduced, while others, such as degeneration and karyolysis, were observed at trace levels (Figure 1f).

The most notable improvement was seen in the group treated with both OO and HXT, which exhibited near-complete recovery. Degeneration, congestion, and rupture were reduced to trace levels, while all other histological abnormalities, including fibrosis, necrosis, inflammatory infiltration, and hemolysis, were absent or within normal limits (Figure 1g). In comparison, the atorvastatin-treated group showed moderate improvement, with several parameters, including fibrosis, degeneration, hemorrhage, and rupture, moderately reduced, while others such as necrosis, NH, and congestion remained at trace levels (Figure 1h).

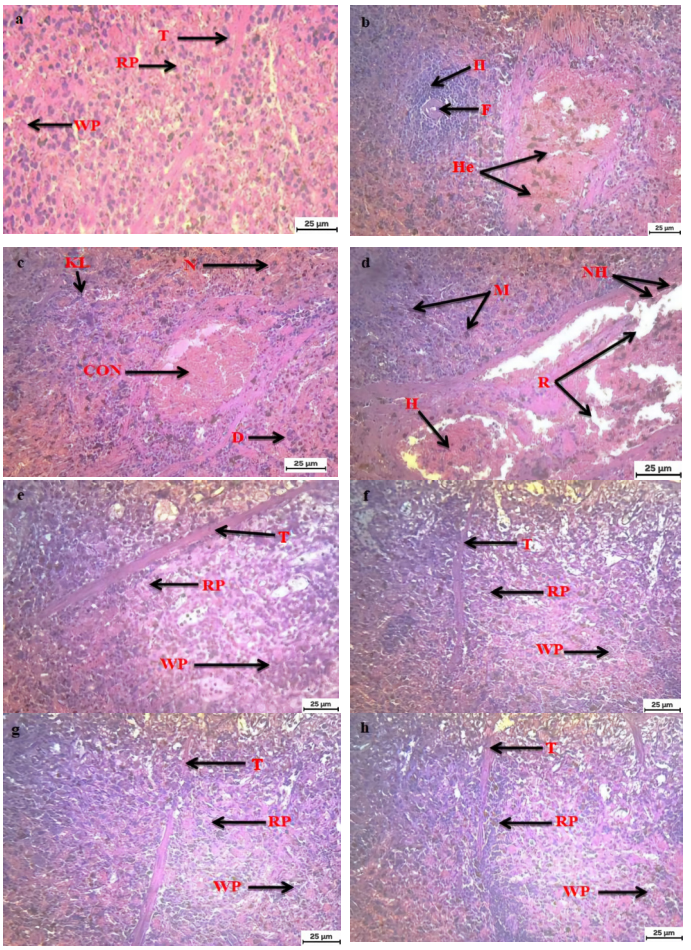

Figure 1: Histological sections of the spleen (H and E, 200X; scale bar 25 µm). Panel (a) represents the control group, while panels (b–d) show spleen sections from the high-cholesterol (HC) diet group. Panel (e) depicts the HC diet group treated with olive oil (OO), panel (f) shows the HC diet group treated with hydroxytyrosol (HXT), panel (g) represents the HC diet group receiving a combination of OO and HXT, and panel (h) shows the HC diet group treated with atorvastatin (ATOR). (H&E 200X. (Scale bar 25 µm).

Table 3: Effect of olive oil active compounds and atorvastatin on histopathological changes in the spleen of the studied groups.

Rupture (R)	Nucleolar Hypertrophy (NH)	Hemolysis (H)	Congestions (Con)	Hemorrhage (He)	Inflammatory Infiltration (II)	Macrophage (M)	Karyolysis (Kl)	Necrosis (N)	Degeneration (D)	Fibrosis (Fi)	Parameters/ Groups
1.0 ± 0.1	0.3 ± 1.0	0.2 ± 0.09	2.0 ± 0.2	0.8 ± 0.05	2.2 ± 0.5	1.9 ± 0.3	0.1 ± 0.0	0.2±0.03	0.9±1.0	1.2±0.3	Control (G0)
6.1 ± 3.5 a	7.0 ± 3.1 a	9.2 ± 3.0 a	6.2 ± 1.1 a	7.2 ± 3.1 a	11.2 ±3.0 a	9.2 ± 1.5 a	2.7 ± 0.1 a	3.8±1.9a	11.1±2.5 a	13.2 ± 3.7 a	GI
3.1 ± 0.3 *b	0.8 ± 0.04 *b	0.5 ± 0.01 *b	3.0 ± 1.2 *b	1.6 ± 0.4 *b	4.1 ± 1.5 *b	2.5 ± 0.05 *b	0.17 ± 0.01 *b	0.6±0.01 *b	2.1±0.3 *b	4.1±1.3 *b	GII
2.5 ± 0.1 *b^	0.9 ± 0.3 *b^	0.6± 0.3 *b^	3.1 ± 0.7 *b^	1.7 ± 0.3 *b^	5.9 ± 1.3 *b^	2.9 ± 0.1 *b^	0.18 ± 0.03 *b^	0.7±0.03 *b^	2.2±0.1 *b^	3.9±1.1 *b^	GIII
2.0 ± 0.2 #bcd	0.6 ± 0.05 #bcd	0.4 ± 0.08 #bcd	2.5± 1.5 #bcd	1.1 ± 0.3 #bcd	6.6 ± 1.8 #bcd	2.2 ± 0.5 #bcd	0.13 ± 0.01 #bcd	0.4±0.03 #bcd	1.4±1.1 #bcd	2.2±0.9 #bcd	GIV
4.0 ± 1.5 abcde	1.0 ± 0.03 abcde	0.7 ± 0.05 abcde	3.9 ± 1.0 abcde	3.1 ± 1.1 abcde	7.9 ± 2.3 abcde	3.1 ± 0.7 abcde	0.2 ± 0.02 abcde	0.8±0.04 abcde	4.9±0.2 abcde	5.9±1.7 abcde	GV
6.466	18.59	41.84	9.020	15.43	13.39	74.02	2817	15.09	51.71	27.18	F
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	p-value

a: statistically highly significant (p<0.001), *: statistically significant (p<0.05), and #: statistically non-significant (p>0.05) compared to control group, b: statistically highly significant (p<0.001) compared to Hypercholesterolemia group (G1), ^: statistically non-significant (p>0.05) and c: statistically highly significant (p<0.001) compared to Hypercholesterolemia and olive oil group (GII), d: statistically highly significant (p<0.001) compared to Hypercholesterolemia and Hydroxytyrosol group (GIII), e: statistically highly significant (p<0.001) compared to Hypercholesterolemia and (Olive Oil and Hydroxytyrosol) group (GIV).

Table 4: Effect of olive oil active compounds and atorvastatin on histopathological changes in the lymph nodes of the studied groups.

Increased macrophage (IM)	Lymphocyte infiltration (LI)	Pulp expansion (PE)	Lymphocyte Degeneration (LD)	Lymphatic Nodule Destruction (LND)	Congestion (Con)	Parameters/ Groups
0.9 ± 0.1	1.9 ± 0.7	1.9 ± 0.3	1.1 ± 0.3	0.2 ± 0.01	1.9 ± 0.3	Control (G0)
10.2 ± 2.1a	7.2 ± 3.0a	9.2 ± 2.4a	4.7 ± 0.4 a	3.9 ±1.9 a	10.1 ± 2.6a	GI
2.1 ± 0.5*b	3.1 ± 0.5*b	2.9 ± 0.5*b	2.4 ± 0.7*b	0.7 ± 0.03 *b	4.9 ± 0.2*b	GII
2.3 ± 0.3*b^	3.0 ± 0.3*b^	2.3 ± 0.1*b^	2.3± 0.3*b^	0.5 ± 0.04*b^	3.7 ± 0.1*b^	GIII
1.7 ± 0.3 #bcd	2.6 ± 0.8 #bcd	2.0 ± 0.5 #bcd	1.9 ± 0.7#bcd	0.3 ± 0.02#bcd	2.4 ± 0.3#bcd	GIV
3.5 ± 0.3abcde	4.9 ± 0.2abcde	3.1 ± 0.7abcde	2.5 ± 0.9 abcde	0.9 ± 0.07abcde	6.0 ± 0.8abcde	GV
70.78	71.06	34.36	20.31	16.33	35.32	F
<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	p-value

a: statistically highly significant (p<0.001), *: statistically significant (p<0.05), and #: statistically non-significant (p>0.05) compared to control group, b: statistically highly significant (p<0.001) compared to Hypercholesterolemia group (G1), ^: statistically non-significant (p>0.05) and c: statistically highly significant (p<0.001) compared to Hypercholesterolemia and olive oil group (GII), d: statistically highly significant (p<0.001) compared to Hypercholesterolemia and Hydroxytyrosol group (GIII), e: statistically highly significant (p<0.001) compared to Hypercholesterolemia and (Olive Oil and Hydroxytyrosol) group (GIV).

Table 3 evaluated histopathological alterations in the spleen across different treatment groups. The hypercholesterolemic group (GI) showed extensive damage, including severe inflammatory infiltration, hemorrhage, and necrosis. Intervention groups showed significant reductions in pathological changes, particularly the GIV group (olive oil and hydroxytyrosol), which had near-normal histological profiles. These findings suggest protective effects of these natural compounds against spleen damage induced by high cholesterol levels. Atorvastatin (GV) also showed moderate improvement but was less effective than GIV in some parameters, highlighting the added value of combined natural treatments.

Histological analysis of lymph nodes

The control group displayed normal lymph node architecture, including a well-defined cortex (C), pulp (P), and lymphatic nodules (LN), as shown in Figure 2a. The Hypercholesterolemic group (HC) showed marked histological alterations, including significant congestion (Con), lymphatic nodule destruction (LND), lymphocyte degeneration (LD), pulp expansion (PE), and sever lymphocyte infiltration (LI), as well as increased macrophage infiltration (IM) (Figure 2b-d).

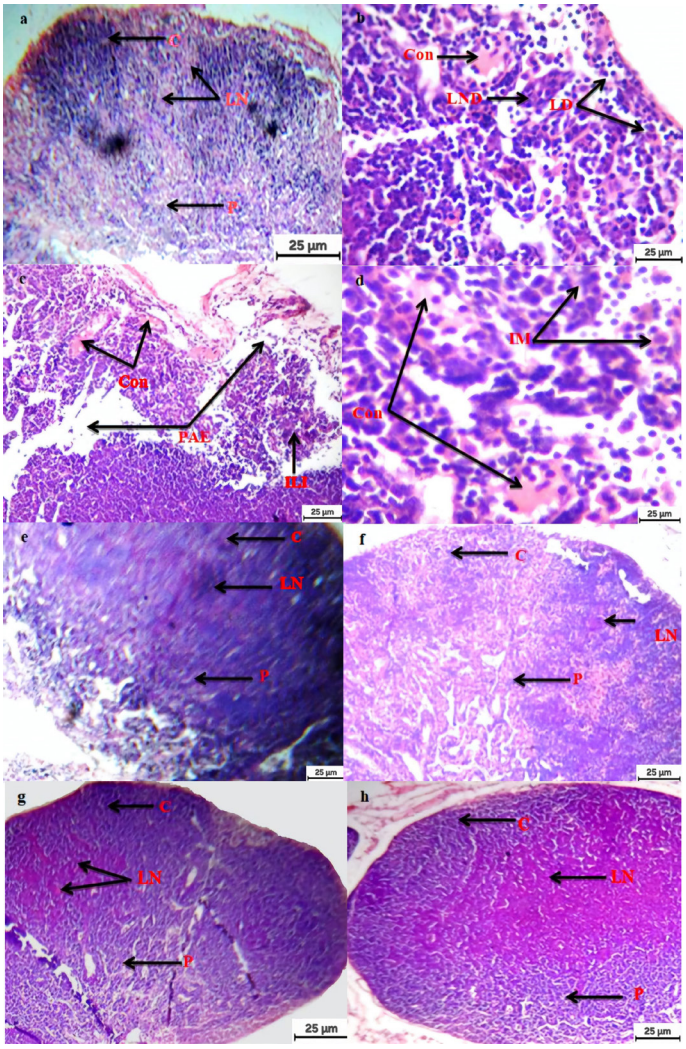

Figure 2: Histological sections of lymph nodes (H and E, 200X; scale bar 25 µm). Panel (a) represents the control group, while panels (b–d) show lymph node sections from the high-cholesterol (HC) diet group. Panel (e) depicts the HC diet group treated with olive oil (OO), panel (f) shows the HC diet group treated with hydroxytyrosol (HXT), panel (g) represents the HC diet group receiving a combination of OO and HXT, and panel (h) shows the HC diet group treated with atorvastatin (ATOR). (H&E 200X. (Scale bar 25 µm).

In the group treated with olive oil alone, moderate improvement was observed in congestion, LD, and PE, while LND, LI, and IM were reduced (Figure 2e). The HXT-only group showed similar patterns of improvement, with most abnormalities reduced to mild or trace levels (Figure 2f).

The group treated with the combined OO and HXT showed the most pronounced recovery. Histological abnormalities were either resolved or minimally observed. Congestion, PE, and LI were reduced to trace levels, while LND, LD, and IM returned to normal (Figure 2g). In contrast, the group treated with atorvastatin exhibited only moderate improvement, with congestion, LD, PE, and LI reduced moderately, while LND and IM were mildly observed (Figure 2h).

Table 4 presented histological evaluation of lymph node damage. Hypercholesterolemic rats (GI) experienced profound disruptions including macrophage increase, lymphocyte degeneration, and congestion. Treatment with olive oil and hydroxytyrosol (GIV) markedly reduced these changes, performing better than individual components or atorvastatin (GV). Inflammatory markers and tissue degeneration were significantly lower in GIV-treated rats, indicating that the combined natural therapy offers superior protective effects in preserving lymph node structure and function under hypercholesterolemic stress. This underscores the potential of dietary antioxidants in mitigating systemic inflammatory impacts of high cholesterol.

DISCUSSION

Our results align with those of Fonollá *et al.* (2020), who reported that 8-week supplementation with olive oil polyphenols reduced serum total cholesterol by 30% and LDL-C by 40% in hypercholesterolemic rats. Derakhshandeh-Rishehri *et al.* (2023) observed a 25% decrease in MDA levels in subjects consuming olive oil rich in polyphenols.

Hyperlipidemia is a major risk factor for cardiovascular diseases, which remain the leading cause of death worldwide. Elevated blood lipid levels, especially cholesterol and triglycerides, contribute to the development of atherosclerosis and its serious complications such as heart attacks and strokes. While pharmacological treatments like statins effectively reduce lipid levels and cardiovascular risk, lifestyle and dietary interventions are essential components of comprehensive management to prevent disease progression and improve overall health (World Health Organization, 2021).

The present study revealed a marked increase in serum levels of leptin, C-reactive protein (CRP), and interleukin-12 (IL-12) in the hypercholesterolemic group (HC) compared to the healthy control group, indicating a systemic inflammatory and metabolic response. Leptin, an adipocyte-derived hormone, plays a key role in regulating energy homeostasis by influencing appetite and energy expenditure. It also serves as a biomarker of fat accumulation and metabolic stress. Elevated leptin levels in this study are consistent with previous research by Padmaja *et al.* (2014), which demonstrated leptin's close association with lipid accumulation and metabolic imbalance. Supporting this, Hashem *et al.* (2021) found that inducing

hyperlipidemia in rats led to a significant rise in circulating leptin levels. Elevated leptin is not merely a marker of obesity; it actively contributes to the pathophysiology of metabolic disorders, such as hypertension and type II diabetes. These conditions are well-established risk factors for cardiovascular diseases, as emphasized by Morris and Edwards (2018). Therefore, the heightened leptin levels observed in our Hypercholesterolemic group further confirm the link between dyslipidemia and systemic metabolic dysfunction, reinforcing leptin's role as both an indicator and a contributor to cardiovascular risk.

In this study, hypercholesterolemic rats exhibited significantly increased levels of MDA, a marker of lipid peroxidation, along with markedly decreased levels of key antioxidant enzymes such as SOD, GPx, and GSH. These changes are indicative of heightened oxidative stress, a common feature in metabolic disorders including hypercholesterolemia. Hydroxytyrosol, a potent phenolic compound found in olive oil, has been widely reported to combat oxidative stress through two primary mechanisms: direct scavenging of reactive oxygen species (ROS) and the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway. The Nrf2 pathway is crucial in upregulating the expression of endogenous antioxidant enzymes, thereby enhancing cellular defense systems. Studies, such as those by Poudyal *et al.* (2017), demonstrated that hydroxytyrosol administration in models of metabolic syndrome led to a significant decrease in MDA levels and a restoration of antioxidant enzyme activities including SOD, GPx, and GSH. In our current findings, the group treated with a combination of olive oil and hydroxytyrosol (GIV) showed a near-complete normalization of oxidative stress markers, closely mirroring healthy control levels. This strongly supports the hypothesis that hydroxytyrosol not only mitigates lipid peroxidation but also reactivates antioxidant pathways, offering a dual protective mechanism against oxidative damage in hypercholesterolemic conditions.

Histopathological examination in this study showed significant structural abnormalities in both the spleen and lymph nodes of rats in the hypercholesterolemic group (G I). These findings are consistent with those reported by Srikakulapu *et al.* (2022), who observed an increase in IgM-secreting B-1b cells in the spleens of hyperlipidemic mice, indicating an immune response linked to lipid dysregulation. The spleen plays a critical role not only in immune surveillance but also in lipid metabolism, acting as a reservoir and processor of immune cells and lipoproteins, as described by Ai *et al.* (2018). Hyperlipidemia-induced oxidative stress has been identified as a major contributor to cellular and tissue damage in the spleen and other lymphoid organs. Dong *et al.* (2022) emphasized that excessive lipid accumulation leads to the generation of reactive oxygen species (ROS), which disrupt cellular integrity and trigger inflammation. Additionally, elevated leptin levels in the hypercholestraemic (GI) group may exacerbate tissue injury. Leptin is known to increase fibrinogen synthesis and blood viscosity, promoting a pro-thrombotic state. It also activates immune cells, including macrophages and natural killer (NK) cells, thereby intensifying local inflammation and tissue remodeling, as reported by García-Estevez *et al.* (2021) and Díaz de León-Guerrero *et al.* (2022). Collectively, these mechanisms explain the extensive histopathological damage observed in the spleen and lymph nodes of hypercholesterolemic rats in the current study.

Treatment with a combination of olive oil and hydroxytyrosol (G IV) led to a significant reduction in serum levels of leptin, C-reactive protein (CRP), and interleukin-12 (IL-12) when compared to the hypercholesterolemic (G I) group. These results are in line with findings by Ahmed *et al.* (2022b), who reported that this combination therapy effectively lowered inflammatory biomarkers and improved lipid metabolism. Beyond biochemical improvements, the combined treatment also resulted in noticeable histological recovery, with reduced tissue damage in the spleen and lymph nodes. The therapeutic benefits observed are largely attributed to the well-established anti-inflammatory and antioxidant properties of olive oil and its phenolic compounds, especially hydroxytyrosol. According to Grases-Pintó *et al.* (2018), hydroxytyrosol can inhibit pro-inflammatory cytokine production and neutralize oxidative stress by scavenging free radicals and activating antioxidant defense mechanisms. Olive oil, particularly extra virgin olive oil, is widely recognized as a functional food due to its high content of monounsaturated fatty acids (especially oleic acid) and an array of bioactive compounds. These constituents contribute significantly to cardiovascular protection and the attenuation of chronic inflammation, as supported by studies from Bucciantini *et al.* (2021) and De Santis *et al.* (2021). The synergistic action of OO and HXT (group IV) observed in this study underscores their potential as natural therapeutic agents in managing hypercholesterolemia and its associated inflammatory and oxidative complications.

The marked histological improvements seen with the combined treatment of olive oil (OO) and hydroxytyrosol (HXT) can be largely attributed to their synergistic impact on lipid metabolism and oxidative stress reduction. This combination was particularly effective in elevating high-density lipoprotein cholesterol (HDL-C) levels while significantly lowering low-density lipoprotein cholesterol (LDL-C) and triglycerides. These changes are crucial, as improved lipid profiles contribute to reduced lipid deposition in tissues and lower risk of inflammatory

damage. According to Carluccio *et al.* (2021), this lipid-modulating effect of OO and HXT also extends to the protection of lipoproteins from oxidative modifications, which is a key factor in preventing atherosclerosis and tissue injury. Furthermore, the phenolic compounds present in olive oil, including hydroxytyrosol, are known to inhibit pro-inflammatory pathways and cytokine release, thereby reducing chronic inflammation. As noted by Visioli *et al.* (2020), these bioactive compounds act at both molecular and cellular levels to suppress inflammatory signaling and oxidative damage. Together, these effects explain the significant reduction in tissue abnormalities observed in the spleen and lymph nodes of rats treated with OO and HXT, highlighting the therapeutic value of this natural compound combination in combating hypercholesterolemia-induced damage.

Atorvastatin treatment (G V) in this study effectively reduced serum levels of leptin, C-reactive protein (CRP), and interleukin-12 (IL-12), aligning with its well-established pharmacological effects. As a statin, atorvastatin not only lowers blood cholesterol levels but also exerts significant anti-inflammatory and antioxidant actions. These findings are consistent with previous studies by Ahmed *et al.* (2020, 2021), which demonstrated that atorvastatin can suppress systemic inflammation and oxidative stress in hyperlipidemic models. Our results further support the therapeutic role of atorvastatin in normalizing oxidative stress markers and improving histological features of affected tissues, as also reported by Ahmed *et al.* (2022a).

A particularly notable effect observed in this study was atorvastatin's ability to reduce leptin levels. This is important because leptin, while primarily known for its role in energy regulation, also functions as a pro-inflammatory cytokine. Elevated leptin levels promote the activation of immune cells, such as macrophages and T cells, while simultaneously inhibiting the proliferation of regulatory T cells that normally suppress excessive immune responses. As described by Lovászi *et al.* (2021) and Oswald *et al.* (2018), this imbalance contributes to chronic inflammation and tissue damage, particularly in the context of metabolic diseases. Therefore, by lowering leptin levels, atorvastatin may help restore immune balance, reduce tissue inflammation, and support cellular homeostasis, which collectively explain the histological improvements seen in treated animals.

CONCLUSIONS AND RECOMMENDATIONS

The study found that both atorvastatin and olive oil polyphenols have strong lipid-lowering and tissue-protective effects. Their combination yielded the best results, significantly reducing LDL-C levels and preserving the structure of the spleen and lymph nodes. A high-cholesterol diet was shown to elevate leptin, CRP, IL-12, and MDA levels, and induced notable tissue damage. However, treatment with olive oil and hydroxytyrosol, especially in combination, effectively improved both biochemical and histological outcomes. This enhanced effect is attributed to the anti-inflammatory and antioxidant properties of olive oil polyphenols. The results suggest that combining natural compounds with conventional drugs may improve the management of hyperlipidemia. Further clinical research is needed to confirm the therapeutic efficacy of olive oil and hydroxytyrosol in human patients with hyperlipidemia. Studies should focus on determining the optimal dosing and duration of olive oil and hydroxytyrosol for maximum efficacy. Additional molecular studies are required to elucidate the precise mechanisms through which these compounds exert anti-inflammatory and lipid-lowering effects.

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NOVELTY STATEMENT

This study uniquely demonstrates the synergistic antioxidant, immunomodulatory, and histoprotective effects of olive oil polyphenols, particularly hydroxytyrosol, when combined with atorvastatin, providing a novel complementary therapeutic strategy for hyperlipidemia management.

AUTHOR'S CONTRIBUTION

All authors contributed equally to the conception, design, data collection, analysis, and interpretation of the study. They all participated in drafting, revising, and approving the final version of the manuscript.

Generative AI and AI-assisted technology statement

AI-assisted tools (ChatGPT) were used only to improve the clarity and language of the manuscript. The authors reviewed, validated, and take full responsibility for the content.

Conflict of interest

The authors have declared no conflict of interest.

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