



Antihyperlipidemic and antioxidant potential of *Olea europaea* L. leaves: An experimental study *in vivo*, *in vitro* and *in silico*

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Abstract:

Hyperlipidemia is an enduring metabolic ailment that affects glucose and lipid processing.

The research objective was to measure the total phenolic, flavonoid, and tannin contents in *Olea europaea* L. leaves and to identify their antioxidant and antihyperlipidemic potential. The study included an *in silico* model of interaction for hydroxytyrosol, oleuropein, and xanthine dehydrogenase. The *in vivo* experiment involved rabbits that received olive leaves (150 mg/kg) and 10 mL of egg yolk as a high-fat diet. At the end of the experimental period, blood samples were tested for lipid profile, and tissue specimens were used for liver histology.

The total phenolic content was 119.84 ± 3.86 mg GAE/g, the total flavonoid content was 2.22 ± 0.07 mg CE/g, and the total tannin content was 21.25 ± 1.24 mg REQ/g dry weight. According to DPPH and FRAP analyses, the antioxidant capacities were 0.34 ± 0.06 μ g/mL and 6.35 ± 0.52 μ mol Fe(II)/g dry weight, respectively. In the experimental animals, *O. europaea* leaves reduced such parameters as total cholesterol, low-density lipoprotein, total triglycerides, total cholesterol vs. high-density lipoprotein, and low-density lipoprotein vs. high-density lipoprotein. The histopathological liver assay showed no signs of tissue damage while the samples obtained from the control group demonstrated steatosis deposits and cellular necrosis. Based on the energy and RMSD results, hydroxytyrosol proved an effective xanthine dehydrogenase inhibition.

These findings constitute a good scientific basis for the complementary future research on the potential of *O. europaea* leaves as ingredients of functional foods or medical drugs.

Keywords: *Olea europaea* L., hyperlipidemia, phenolic compounds, antioxidant activity, antihyperlipidemic activity, xanthine dehydrogenase

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INTRODUCTION

Hyperlipidemia refers to increased presence of one or more lipid types in the bloodstream. This condition is evident through high cholesterol, sometimes accompanied by triglycerides [1–3]. Hyperlipidemia can be classified into two groups as either primary (familial) or secondary (acquired) hyperlipidemia. Primary hyperlipidemia originates from various genetic disorders while secondary hyperlipidemia typically arises as a result of inadequate diet, certain medications (amiodarone, glucocorticoids, etc.), hypothyroidism, uncontrolled diabetes, and unhealthy lifestyle [4]. The global incidence of hyper-

lipidemia has been progressively rising, which is commonly attributed to lifestyle and dietary factors [5].

As a multifaceted and enduring metabolic ailment, hyperlipidemia encompasses disruptions in glucose and lipid processing, along with broader systemic imbalances within the body. These imbalances include thickening of blood vessel walls, obesity, and high blood sugar [6]. Hyperlipidemia can directly trigger metabolic disorders, thus causing significant disruptions in metabolism and exerting adverse effects on intestinal well-being [7, 8]. Hyperlipidemia could be linked to an increased cumulative risk of myocardial infarction, ischemic stroke, and

premature mortality [9]. High cholesterol is a significant factor that contributes to a third of global ischemic (coronary) heart disease cases. In total, high cholesterol is accountable for 2.6 million deaths, making up 4.5% of the total mortality [10, 11].

Managing hyperlipidemia includes such strategies as dietary regulation, physical activity, and medication. According to Gold *et al.*, only a quarter of adults with significantly elevated low-density lipoprotein cholesterol (LDL-C) managed to attain the target reduction suggested by LDL-C guidelines [12]. The Spanish COHORT Familial Hypercholesterolemia Study provided individuals with familial hypercholesterolemia with lipid-lowering therapy; however, only a minority of 11.2% patients achieved the treatment objective of reducing low-density lipoprotein cholesterol below 100 mg/dL [13]. Several studies showed that hypercholesterolaemia correlates with the generation of reactive oxygen species that are known to cause oxidative damage to human cells. This process can be inhibited by antioxidant agents, e.g., polyphenols. They inhibit the propagation of free radicals either directly or indirectly, i.e., by reacting with enzymes involved in the production of reactive oxygen species [14].

The past few decades have seen a significant upsurge in alternative anti-hypercholesterolaemia remedies, particularly herbal medicines and their supplements [15]. Plants tend to yield compounds with fewer toxic side effects than synthetic medications, which makes them prospective raw materials for novel therapeutic agents [16]. Ameeruddy *et al.* conducted a major review of popular herbal remedies utilized worldwide to manage high cholesterol [17]. The review covered a total of 174 surveys from 2001–2020 and revealed records of 390 plant species, encompassing 109 families and 294 genera. The studies were published by research teams from 37 countries and involved leaves (29%) and fruit (15%) as anti-hypercholesterolaemia remedies. Many of them featured *Olea europaea* L., commonly known as the olive tree. This species was reported across six countries: Algeria, Argentina, Greece, Palestine, Portugal, and Turkey. Olive (*O. europaea*) leaves contain a plethora of potentially bioactive compounds with antioxidant, antihypertensive, antiatherogenic, hypoglycemic, anti-cancer, and anti-inflammatory properties [18, 19].

Presumably, olive leaf extracts owe their biological properties to such potentially bioactive compounds as oleuropein and hydroxytyrosol [20, 21].

This article introduces the anti-hypercholesterolaemia potential of *O. europaea* leaves. We measured the total phenolic, flavonoid, and tannin contents to define their antioxidant and antihyperlipidemic potential. In literature sources, we did not find research on the antihyperlipidemic activity of *O. europaea* leaves using a high-fat diet and induced hypercholesterolaemia in rabbits as model animals. This research also featured an *in silico* interaction model of such phenolic compounds as hydroxytyrosol and oleuropein with xanthine dehydrogenase, which is a key enzyme in generating reactive oxygen species.

STUDY OBJECTS AND METHODS

Collecting the plant material. The olive leaves (*Olea europaea* L.) of a local Hamri variety were harvested in the Skikda region, North-East of Algeria, in early 2022. After a thorough cleaning, the leaves were dried at 40°C for 10 days to be ground to fine powder. The resulting powder was kept in a hermetically sealed bottle at room temperature in a dry and dark place until further use.

Preparing the extract. For extraction, we used a method described by Bhatia *et al.* [22]. After soaking 20 g of the olive leaf powder in 100 mL of 70% methanol at room temperature for 24 h, we filtered the mix and performed a triplicate maceration with solvent renewal in order to extract the maximum of the bioactive product. Then, we removed methanol in a rotary vacuum evaporator at 40°C and stored the resulting extract in airtight bottles at 4°C until use.

Total phenolics assay. To assess the total phenolic content, we applied the spectrophotometric method as described by Al-Farsi *et al.* [23]. In line with the procedure, we added 200 µL of the extract to 1.5 mL of the Folin-Ciocalteu reagent. After mixing and incubating the solutions in the dark for 5 min, we added 1.5 mL of sodium bicarbonate (60 g/L) to the reaction medium. Following 90 min of incubation at room temperature, we measured the absorbance with an ultraviolet-visible spectrophotometer at 725 nm against the blank without extract. The phenolic content was expressed as mg GAE/g dry weight, i.e., milligrams of gallic acid equivalent per 1 g dry weight.

Total flavonoids assay. Here, we followed the protocol described by Biglari *et al.* [24]. To assess the total amount of flavonoids in the extract, we added 4.0 mL of distilled water and 1 mL of the extract to 0.3 mL of 5.0% sodium nitrite (NaNO₂) and 0.3 mL of 2.0% aluminum chloride (AlCl₃) dissolved in methanol. After 5-min incubation at room temperature, we added 2 mL of 1.0% sodium hydroxide (NaOH) dissolved in methanol. The mix was then diluted to 10 mL with distilled water, and the absorbance was measured at 510 nm against the blank. The flavonoid content was expressed as mg CE/g dry weight, i.e., milligrams of catechin equivalent per 1 g dry weight.

Total tannins assay. To determine the total amount of tannins, we used the acidic vanillin method as described by Qaisar *et al.* [25]. We prepared the vanillin reagent by mixing the same volumes of 8% (v/v) HCl, 37% (v/v) methanol, and 4% vanillin in methanol (w/v). After storing the mix at 30°C, we added 200 µL of the extract to 1000 µL of the vanillin reagent. The mix was agitated and incubated in the dark at 30°C for 20 min. To measure the absorbance at 500 nm, we used a blank that consisted of a mix of methanol (37%) and HCl (8%) in equal volumes. The results were expressed as mg QE/g dry weight, i.e., milligrams of quercetin equivalent per 1 g dry weight.

Antioxidant activity. DPPH assay. As recommended by Blois, we measured the antioxidant potential of the olive leaf extract to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [26]. We mixed 60 µL of different concentrations of the extract with 1500 mL of the DPPH solution (6×10⁻⁵ M). After 30 min of incuba-

tion at room temperature, we monitored the absorbance at 517 nm. The percentage inhibition (I, %) of the DPPH radical was calculated according to the Equation below:

$$I = (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}}) \times 100$$

where A_{blank} is the absorbance of the control; A_{sample} is the absorbance of the test extract with the DPPH solution.

The same procedure was repeated for butylated hydroxytoluene solutions as positive control. The antioxidant activity of the extract was expressed as IC_{50} , which represents the concentration ($\mu\text{g/mL}$) of the extract required to scavenge 50% of the DPPH free radical.

FRAP assay. The ferric-reducing antioxidant power (FRAP) of the methanolic extract of *O. europaea* leaves was measured according to the method described by Benzie & Strain [27]. In line with the protocol, we added 20 μL of the extract to 1.5 mL of the FRAP reagent. The obtained blue solutions remained at room temperature at 37°C for 20 min for the absorbance to be measured at 593 nm. We used ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) concentrations (100–2000 mmol/L) to calibrate the standard curve. The ferric-reducing antioxidant power was expressed as mmol Fe(II) per 1 g dry weight.

Experimental animals. Ethic. All the experiments on animals were confirmed and approved by the Department of Natural and Life Sciences, University of Skikda, Algeria. The experiments were conducted in line with the Guide for the Care and Use of Laboratory Animals.

Animals. The research involved healthy male rabbits (*Oryctolagus cuniculus* L.). The animals were eight weeks old and weighed 1.6–2.5 kg. They were obtained from a local supplier (Hama Bouziane, Constantine, Algeria). All the animals were kept under standard environmental conditions: 12 h light/12 h dark cycle, $20 \pm 2^\circ\text{C}$. They had free access to tap water and food. The animals were quarantined for ≥ 10 days before the experiment.

The **antihyperlipidemic activity of *O. europaea* leaves** was measured based on the method proposed by Djerrou [28]. We divided a total of 25 rabbits randomly into five experimental groups with five animals in each ($n = 5$):

- group 1 represented normal control and received a standard diet;
- group 2 were subjected to oral feeding (gavage) with *O. europaea* leaves (150 mg/kg);
- group 3 received standard diet and 10 mL of egg yolk, i.e., a high-fat diet;
- group 4 received atorvastatin (2.5 mg/kg) followed by 10 mL of egg yolk after 30 min; and
- group 5 animals were fed with olive leaf powder (150 mg/kg) followed by 10 mL of egg yolk after 30 min.

Assay of plasma lipid and hepatic parameters. At the end of the experimental period (45 days), the rabbits were sacrificed to collect blood samples. Within 1 h, the samples were sent to a diagnostic laboratory for a lipid profile test using a BS-240 Mindray autoanalyzer. The

test involved the following parameters: total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total triglycerides, total cholesterol vs. high-density lipoprotein, and low-density lipoprotein vs. high-density lipoprotein.

Histopathological study. The livers were washed with ice normal saline and fixed in a 10% formalin solution for histological assessment. The sections were assessed on hematoxylin and eosin to be examined with optic microscopy (Carl Zeiss MicroImaging GmbH, Germany).

In silico study of the interaction of xanthine dehydrogenase and phenolic compounds. We used a method described by Patel & Kukol to study the interaction between xanthine dehydrogenase and phenolic molecules [29]. Xanthine dehydrogenase is a key enzyme in generating reactive oxygen species while hydroxytyrosol and oleuropein are the major phenolic compounds in *O. europaea* leaves.

Preparing protein. The structure of xanthine dehydrogenase was downloaded from the Protein Data Bank Archive (www.pdb.org) with PDB ID: 1N5X at 2.80 Ångström resolutions. Then, we used the AutoDockTools software to add Gasteiger-type atomic charges and hydrogen atoms. The structures were saved in the PDBQT format.

Preparing ligand. This part of the research involved two phenolic compounds, i.e., hydroxytyrosol and oleuropein. We downloaded the ligands from the PubChem site (<https://pubchem.ncbi.nlm.nih.gov>) in the SDF format to be reformatted into PDB using the PyMOL software. To increase the energy evaluation of the system, the receptor was immersed in a three-dimensional grid, which largely encompassed the active site of the protein and allowed the ligand to rotate freely in this site. The center of the grid box was determined by the coordinates X, Y, and Z with the dimensions of 3 Ångström. The spacing of the grid was fixed at 1 Ångström. The grid box was then centered on the ligand, its dimensions being proportional to the size of all the ligands in the study.

Calculating root mean square deviation. The reliability of a docking program is evaluated in terms its ability to reproduce protein-ligand complexes. Using the docking program, we determined the root mean square deviation (RMSD) between the conformation and the orientation of the ligand. The place it occupied in the experimental complex had to be as small as possible. The allowed ratio was a maximal difference of 2 Ångström. We used Discovery Studio 4.0 Client to calculate the root mean square deviation while AutoDock Vina served to determine the root mean square deviation and perform the visual analysis.

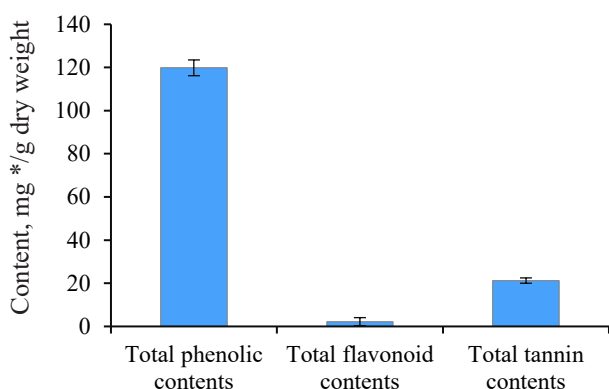
Statistical analysis. The results were given as mean \pm standard deviation. The statistical analysis involved SPSS Statistics 16.0 (Chicago, USA). The analysis of variance (ANOVA) made it possible to identify the differences in the mean values. We applied the Tukey's test to register statistically significant difference. The significance level was $p < 0.05$.

RESULTS AND DISCUSSION

Bioactive content. Figure 1 shows that the methanolic extract of *Olea europaea* L. leaves was rich in phenolic compounds. The total phenolic, flavonoid, and tannin contents were 119.84 ± 3.65 mg GAE/g dry weight, 2.22 ± 1.86 mg CE/g dry weight, and 21.25 ± 1.24 mg REQ/g dry weight, respectively.

Our results were in agreement with those obtained by Ines *et al.*, who reported the phenolic contents of olive leaf extract (China) as 151.74 mg GAE/g [30]. Bixia *et al.*, who worked with Tunisian *O. europaea* leaves, reported a higher total phenolic content of 905.96 mg GAE/g [31]. Results of Turkish and Saudi Arabian researchers for the total phenols were lower than ours, namely 37.8 and 45.48 mg GAE/g, respectively [32, 33]. Sirajudheen *et al.*, who also worked with *O. europaea* leaves from Saudi Arabia, reported the total flavonoid content between 3.11 ± 0.67 and 6.44 ± 0.12 mg CE/g [33]. Our findings more or less coincided with these results. However, Nashwa & Abdel-Aziz reported the total flavonoid content as 21.45 mg EQ/g for the methanolic extract of olive leaves from Egypt [34]. These results exceeded those obtained in our study. To our best knowledge, no data regarding the total tannin contents in *O. europaea* leaf extracts have been published so far.

Chromatographic separation of aqueous extracts from Egyptian genotypes of the *O. europaea* plant revealed



*Contents are represented as follows: total phenolics – milligram gallic acid equivalent per 1 g dry weight (mg GAE/g dry weight); flavonoids – milligram catechin equivalent per g dry weight (mg CE/g dry weight), and tannins – milligram quercetin equivalent per g dry weight (mg REQ/g dry weight)

Figure 1 Bioactive contents of the extract of *Olea europaea* L. leaves

Table 1 Antioxidant activities of *Olea europaea* L. leaf extract

	DPPH IC ₅₀ , μg/mL	FRAP, moles Fe(II)/g DW
<i>Olea europaea</i> L. leaves	0.34 ± 0.06^b	6.35 ± 0.52
Butylated hydroxytoluene	0.18 ± 1.50^a	–

a–b: If values (mean \pm standard deviation, n = 3) in the same column have different letters, they are significantly different ($p < 0.05$). DPPH – 2,2-diphenyl-1-picrylhydrazyl; FRAP – ferric-reducing antioxidant power; IC₅₀ – the extract concentration that scavenges 50% DPPH

the presence of gallic acid, hydroxytyrosol, catechol, *p*-hydroxy benzoic acid, caffeine, vanillic acid, caffeic acid, syringic acid, oleuropein, vanillin, *p*-coumaric acid, ferulic acid, rutin, ellagic acid, benzoic acid, *o*-coumaric acid, salicylic acid, and cinnamic acid [35, 36]. Several studies identified oleuropein as a primary compound in *O. europaea*. These phytochemical components are known for their potent biological activities [37, 38]. Sirajudheen *et al.* mentioned 6-C-glucopyranosyl-8-cxylopyranosylchrysoeriol, quercetin 3-galactoside-7-rhamnoside, isovitexin, 6-hydroxyluteolin 5-rhamnoside, melanoxetin, calomelanol D-1, monotropein, tephrocin, robinetin 3-rutinoside, isovitexin 7-O-rhamnoside, isovitexin, and kaempferol 3-(2''-(Z)-*p*-coumaroylglucoside) as the dominant flavonoids in the methanolic extract of *O. europaea* leaves [33]. The phytochemical composition of *O. europaea* leaf extracts depends on the geographic origin, climate, variety, growing conditions, maturity, season, soil, cultural specifics, processing methods, and solvent [39].

Antioxidant activity. Table 1 summarizes the values of the antioxidant activities of *O. europaea* leaves based on DPPH and FRAP assays. The butylated hydroxytoluene, which was used as standard, had a stronger capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (0.18 ± 1.5 μg/mL) than the extract of *O. europaea* leaves (0.34 ± 0.06 μg/mL). As for the ferric-reducing antioxidant power, the olive extract demonstrated a rather high antioxidant potential of 6.35 ± 0.52 moles Fe(II)/g dry weight, as well as an ability to reduce the ferric iron (Fe⁺³) to ferrous iron (Fe⁺²).

Mansour *et al.* studied three *O. europaea* cultivars from Egypt, namely, *picual*, *tofahi*, and *shemlali* [36]. Their IC₅₀ ranged from 48.14 ± 0.15 to 56.00 ± 0.13 μg/mL. As for the Turkish variety, its IC₅₀ was 3.80 mg/mL [40].

Our findings agree with those reported by Cheurfa *et al.*, whose team studied the antioxidant activity of the ethanolic and aqueous extracts of *O. europaea* leaves cultivated in Chlef, Algeria [41]. Their FRAP assay rendered the values of 07.53 ± 0.06 and 4.01 ± 0.01 moles Fe(II)/g dry weight, respectively. Presumably, *O. europaea* leaves owe their antioxidant properties to their high oleuropein [42]. This natural phytochemical protects human cells against free radicals that react with cellular molecules of fats, proteins, and DNA, thus producing oxidative damage [43]. The antioxidant potencies of oleuropein may be due to its capacities to chelate copper and iron. Ions of these metals generate reactive oxygen species, which are known to trigger cancer, hypertension, cardiovascular diseases, and inflammatory disturbances [37].

Antihyperlipidemic activity of *Olea europaea* L. leaves. The concentration of total cholesterol significantly increased ($p < 0.05$) in the group of rabbits fed with egg yolk (1.07 ± 0.306 g/dL) compared to the untreated control rabbits (0.49 ± 0.72 g/dL). The concentration of total cholesterol also increased compared to the group of rabbits treated with olive leaf powder (0.67 ± 0.08 g/dL). However, both groups fed with egg yolk and treated with either atorvastatin (0.71 ± 0.12 g/dL) or olive leaf

powder (0.76 ± 0.08 g/dL) showed a significant decrease ($p < 0.05$) compared to Group 3, which received a standard diet and 10 mL of egg yolk. We detected a significant increase ($p < 0.05$) in high-density lipoprotein in the rabbits that received a high-fat diet (0.482 ± 0.070 g/dL) compared to the control (0.27 ± 0.06 g/dL) and Group 2 (0.30 ± 0.03 g/dL), which received olive leaves. Furthermore, we observed a significant decrease in Group 4 (0.35 ± 0.71 g/dL), treated with atorvastatin and egg yolk, compared to Group 3, which received olive leaves and egg yolk. However, the group of rabbits fed with olive leaves showed some decrease (0.44 ± 0.05 g/dL), although it was not significant, compared to Group 3.

The concentration of low-density lipoprotein-cholesterol dropped ($p < 0.05$) in Group 3 (0.44 ± 0.21 g/dL) compared to Group 2 (0.09 ± 0.05 g/dL) and increased insignificantly compared to Group 1 (0.26 ± 0.25 g/dL), which received a standard diet. In contrast, Groups 4 and 5, which both received egg yolk and atorvastatin or olive leaf powder, showed an insignificant decrease ($p > 0.05$) compared to Group 3, which received egg yolk and a standard diet, with respective levels of 0.22 ± 0.05 and 0.24 ± 0.08 g/dL.

The concentration of total triglycerides increased insignificantly in Group 3 (0.76 ± 0.27 g/dL) compared to Group 2 (0.61 ± 0.09 g/dL) and Group 1 (0.70 ± 0.18 g/dL). However, the triglyceride level decreased insignificantly in Group 4 (0.66 ± 0.14 g/dL) and the group fed with olive leaf powder (0.55 ± 0.22 g/dL) compared to Group 3, which received egg yolk and a standard diet.

The results showed no significant increase ($p > 0.05$) in the ratio of total triglycerides and high-density lipoprotein in Group 3 (2.10 ± 0.45 g/dL), compared to control (1.91 ± 0.60 g/dL). However, we observed no significant decrease in the group of rabbits fed with olive leaves (1.75 ± 0.35 g/dL) and atorvastatin (2.01 ± 0.25 g/dL), compared to Group 3. We also detected no significant increase in the low-density lipoprotein vs. high-density lipoprotein ratio in Group 3 (0.89 ± 0.32 g/dL), compared to Group 2 (0.33 ± 0.21 g/dL). No significant decrease ($p > 0.05$) was registered in Group 3 compared to Group 1 (1.03 ± 1.10 g/dL). Similarly, we found no significant decrease ($p > 0.05$) in the low-density lipoprotein vs. high-density lipoprotein ratio in Group 4 (0.46 ± 0.16 g/dL) and Group 5 (0.57 ± 0.23 g/dL), which received atorvas-

tatin or olive leaves with egg yolk, compared to Group 3, which received a standard diet and 10 mL of egg yolk.

Epidemiological data correlate a high consumption of plant extracts with a lower risk of several cardiovascular and degenerative diseases, hence the increasing scientific interest in their therapeutic properties as sources of health-promoting phytochemical molecules. Several experiments on people, mice, and rats reported that the extract of *O. europaea* leaves improved the plasma lipids profile (Table 2). However, to our best knowledge, no publications have so far reported the antihyperlipidemic activity of *O. europaea* leaves using a high-fat diet and induced hypercholesterolaemia in rabbits as model animals.

Our findings were in line with several previous studies. For instance, Cheurfa *et al.* studied administered aqueous extract of *O. europaea* leaf cultivated in Chlef, Algeria, on serum total cholesterol, triglycerides, high-density lipoproteins, low-density lipoproteins, and very low-density lipoproteins in hypercholesterolemic mice [41]. The mice treated with the extracts showed lower levels of total cholesterol, low-density lipoproteins, and triglycerides. In addition, they reported rutin and luteolin to be naturally present in the leaves of *O. europaea*. These substances were anchored against HMG-CoA reductase, the enzyme that limits cholesterol metabolism.

Atorvastatin is a synthetic lipid-lowering agent; it is a competitive inhibitor of HMG-CoA-reductase. It reduces total cholesterol, triglyceride levels, and low-density lipoprotein cholesterol [44].

Jang *et al.* reported that such phenolic compounds as gallic acid and linoleic acid, as well as their mixes, improved the serum lipid profile in hypolipidemic C57BL/6 mice by decreasing serum triglyceride and low-density lipoprotein cholesterol [45].

Hadrich *et al.* studied the effect of orally-administered oleuropein (50 mg/kg) on adiponectin secretion [46]. Oleuropein exerts hypocholesterolemic effect by inhibiting peroxisome proliferator-activated receptor γ , sterol regulatory element-binding protein-1c, and fatty-acid synthase expression. The team reported a protective effect of oleuropein and hydroxytyrosol derived from olive leaves on high-fat diet-induced lipid metabolism disorders. These phenolic compounds exerted their hypolipidemic and hepatoprotective effects by improving

Table 2 Effect of *Olea europaea* L. on plasma profile lipids

Variables, mg/dL	Group 1 (standard diet)	Group 2 (olive leaves)	Group 3 (standard diet + yolk)	Group 4 (atorvastatin + yolk)	Group 5 (olive leaves + yolk)
Total cholesterol	0.49 ± 0.72^a	0.67 ± 0.08^a	1.07 ± 0.30^b	$0.71 \pm 0.12^{a,c}$	$0.76 \pm 0.08^{a,c}$
High-density lipoprotein	0.27 ± 0.06^a	0.30 ± 0.03^a	0.48 ± 0.07^b	$0.35 \pm 0.71^{a,b,c}$	0.44 ± 0.05^b
Low-density lipoprotein	0.26 ± 0.25^a	0.09 ± 0.05^a	$0.44 \pm 0.21^{a,b}$	$0.22 \pm 0.05^{a,b}$	$0.24 \pm 0.08^{a,b}$
Total triglycerides	0.70 ± 0.18^a	0.610 ± 0.096^a	0.76 ± 0.27^a	0.66 ± 0.14^a	0.55 ± 0.22^a
Total cholesterol vs. high-density lipoprotein cholesterol	1.91 ± 0.60^a	2.23 ± 0.16^a	2.10 ± 0.45^a	2.01 ± 0.25^a	1.75 ± 0.35^a
Low-density vs. high-density lipoprotein	1.03 ± 1.10^a	0.33 ± 0.21^a	0.89 ± 0.32^a	0.46 ± 0.16^a	0.57 ± 0.23^a

antioxidative defense system and blocking the expression of proteins involved in inflammation and liver damage [30].

Hepatoprotective activity of *Olea europaea* L. leaves. Figure 2 illustrates the results of the histopathological tests. The morphological features of the liver in the positive control group showed a normal architecture of hepatocytes (Fig. 2a). The histology of the rabbits that received a standard diet together with olive leaves also demonstrated a normal architecture of hepatocytes (Fig. 2b). The microscopic observation of the liver obtained from the rabbits that received a hyper-lipid diet (control group) revealed some changes in the tissue architecture, namely, steatosis deposits, bloating, and cellular necrosis (Fig. 2c). However, the histopathological architecture of the liver sections obtained from the rabbits treated with the olive leaves (test group, Fig. 2d) and atorvastatin (reference group, Fig. 2e) demonstrated no histological changes in comparison with Group 3, which received a standard diet with egg yolk.

Increasing incidences of some chronic diseases, including hyperlipidemia, have raised awareness regarding the importance of diet. Numerous investigations determined that animals fed with high-fat and high-cholesterol feeds developed nonalcoholic fatty liver diseases. This pathology is characterized by steatosis, necroinflammation, ballooning, and fibrosis. Gaube *et al.* reported that the oral administration of ethanolic olive leaf extract induced no changes in histopathology, biochemical profile, and hematological parameters after single or repeated doses in a rat model [47]. According to Taamalli *et al.*, the attenuation of hepatic tissues correlated quite well with the biochemical contents [48]. They explained the hepatoprotective potentials of *O. europaea* leaves by the high phenolic contents that were able to reduce the inflammatory and oxidant disorders in hepatic cells.

The hepatoprotective activity of *O. europaea* leaves has been elucidated against hepatic damages induced by cadmium, carbon tetrachloride (CCl₄), and diazinon. Jemai *et al.* evaluated the hepatoprotective potential of oleuropein at 16 mg/kg body weight against cadmium-induced hepatotoxicity in mice [49]. Orally-administered oleuropein restored significantly such biomarkers of liver injury as alanine transaminase, aspartate transaminase, lactate dehydrogenase, and phosphatase alkaline,

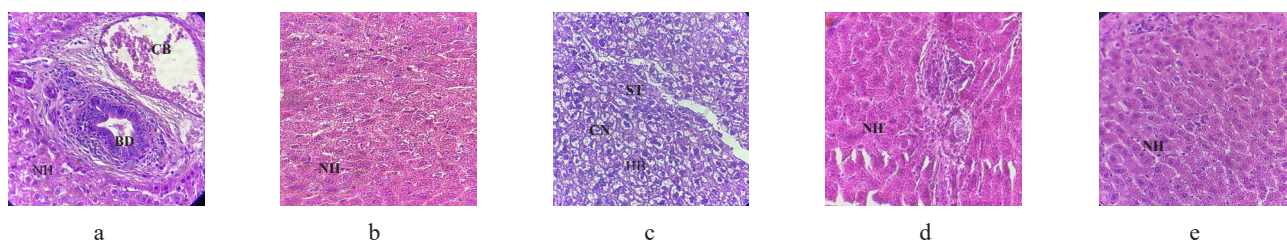
compared to the animals that received cadmium alone. The histological and immunohistochemical tests showed a significant suppression of the inflammation scores, as well as the oxidative damage induced by cadmium in hepatic tissues. Rats pretreated with olive leaf extracts showed less oxidative damage in ischemic and non-ischemic parts of liver, as well as a significant improvement in physiological and histopathological disorders. The predominant mechanism of hepatoprotective action is due to the high antioxidant potential and the capacity to scavenge free radicals [50]. Vidičević *et al.* studied the protective mechanisms of dry olive leaf extract in hepatotoxic rats treated with carbon tetrachloride (CCl₄) [51]. The simultaneous treatment with CCl₄ and the extract of *O. europaea* leaves significantly reduced the expression of protein kinase activated by adenosine monophosphate (AMPK) and inhibited the expression of autophagy-related protein LC3II compared with the control group. Omagari *et al.* confirmed the protective effect of oleuropein in reducing numerous hepatic genes involved in the production of reactive oxygen species and the regulation of cholesterol or lipid metabolism [52]. These effects are due to the properties of antioxidants that act as reducing agents by donating hydrogen and quenching singlet oxygen or by acting as chelators and trapping free radicals.

Interaction *in silico* between xanthine dehydrogenase and phytochemicals. Binding free energy. In this test, oleuropein formed the most stable complex with xanthine dehydrogenase with binding free energy (ΔG) of -7.5 kcal/mol, followed by hydroxytyrosol with an energy of -6.4 kcal/mol.

Interaction between hydroxytyrosol and xanthine dehydrogenase. A three-dimensional visualization of the interactions between the active site of xanthine dehydrogenase and hydroxytyrosol showed that the amino acids involved in the interaction were represented by leucine 788 and serine 1064 (Fig. 3).

According to the Discovery Studio model, hydroxytyrosol penetrated well into the active site of xanthine dehydrogenase by forming different pi-cation, pi-alkyl, hydrogen, pi-anion, and hydrogen carbon interactions (Table 3).

Interaction between oleuropein and xanthine dehydrogenase. A three-dimensional visualization of the interactions between the active site of xanthine



NH – normal hepatocytes, BD – bile duct, CB – capillary blood, HB – hepatocyte ballooning, ST – steatosis deposit, CN – cellular necrosis

Figure 2 Light microscopy of rabbit liver tissue stained with hematoxylin and eosin ($\times 400$): a – Group 1, standard diet; b – Group 2, olive leaves; c – Group 3, standard diet and yolk; d – Group 4 atorvastatin and yolk; e – Group 5, olive leaves and yolk

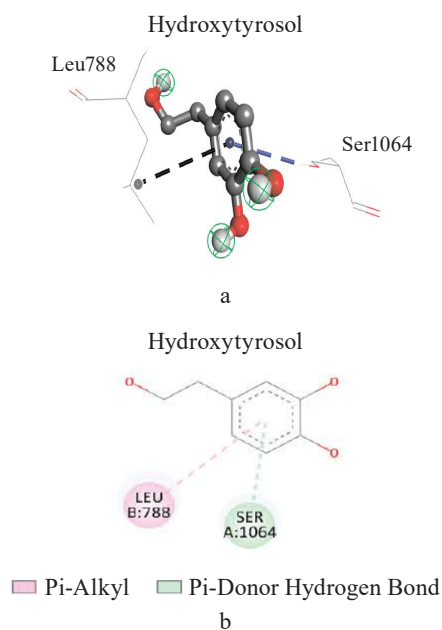


Figure 3 Three-dimensional (a) and two-dimensional (b) visualizations of interactions between the active site of xanthine dehydrogenase and hydroxytyrosol

Table 3 Interactions between hydroxytyrosol and xanthine dehydrogenase

Residues involved in the interaction	Type of interaction	Distances (Ångstrom)
Leucine 788	Pi-Alkyl	2.88938
Serine 1064	Pi-donor-hydrogen-bond	4.00545

Table 4 Interactions between oleuropein and xanthine dehydrogenase

Residues involved in the interaction	Type of interaction	Distances (Å)
Asparagine 595	Unfavorable acceptor-acceptor	2.24364
Proline 597	Carbon hydrogen bond	3.19379
Proline 753	Conventional hydrogen bond	2.41202
Lysine 754	Conventional hydrogen bond	2.43452
Arginine 37	Conventional hydrogen bond	3.06085
Histidine 821	Conventional hydrogen bond	2.31595
Glycine 588	Conventional hydrogen bond	2.34949

dehydrogenase and oleuropein showed that the amino acids involved in the interaction were represented by glycine 38, glycine 588, glycine 35, lysine 95, and proline 753 (Fig. 4).

According to the Discovery Studio model, oleuropein penetrated well into the active site of xanthine dehydrogenase by forming hydrogen and carbon hydrogen bonds (Table 4).

Reliability testing of the molecular docking program. Root mean square deviation. Interaction mode prediction consists of determining the correct positioning of the ligand in relation to its receptor. The ability of a program to perform this work is usually judged

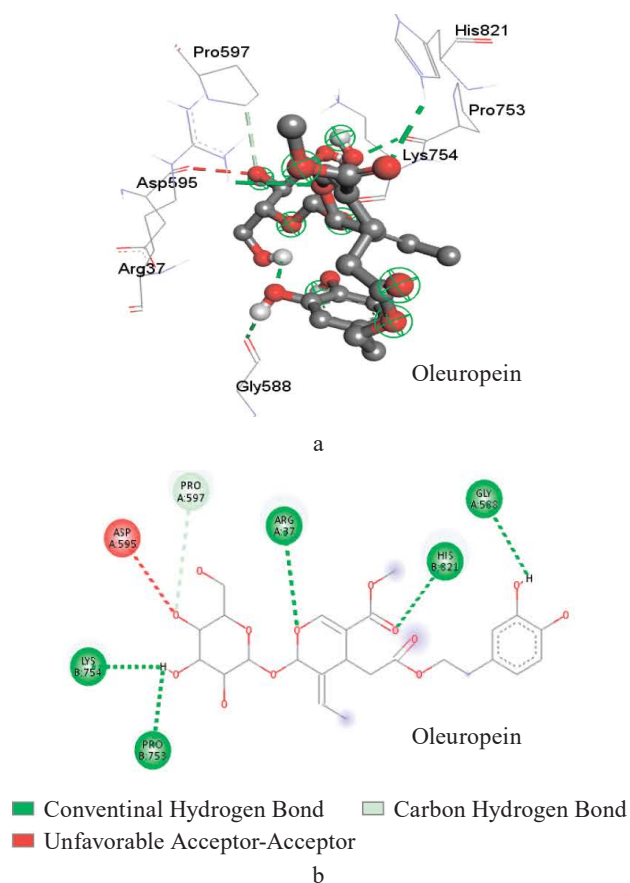


Figure 4 Three-dimensional (a) and two-dimensional (b) visualization of interactions between the active site of xanthine dehydrogenase and oleuropein

Table 5 Root mean square deviation values of ligand interaction with xanthine dehydrogenase

Ligands	Root mean square deviation (Ångström)
Hydroxytyrosol	0.994
Oleuropein	2.126

by means of the root mean square deviation (RMSD) of the model designed by the Vis-à-vis software regarding the structure of the reference (co-crystallized) ligand. The permissible limit is 2Å , beyond which the prediction is considered inadequate [53]. The enzyme complex used in this study is xanthine dehydrogenase (pdb: 1n5x). This procedure is followed by extracting the co-crystallized ligand from the given target and repositioning it by docking in the active site. The root mean square deviation of the best exposure of the reference ligand (after docking) is calculated with its crystallographic binding mode (before docking). The RMSD value of hydroxytyrosol of the software-designed model does not exceed 2Å (Table 5).

Visual analysis is an essential step in judging the results described by the numerical value of the root mean square deviation. Visualization of the complexes selected, namely, xanthine dehydrogenase (pdb: 1n5x) and ligands made in the PyMOL molecular visualization system showed that the ligands predicted by

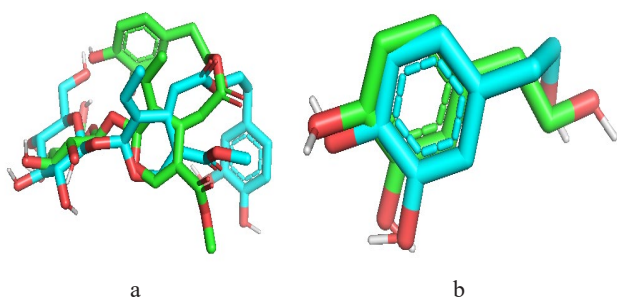


Figure 5 Superposition of inhibitors (PyMOL) to calculate the root mean square deviation: a – hydroxytyrosol; b – oleuropein

AutoDock-Vina and the reference ligand were well positioned. Indeed, these results confirmed the root mean square deviation values.

The design of new plant-based computer-aided products (*in silico* study) is a relatively recent method for high-throughput screening of an extensive chemical database. It produces results for major compounds in less time and at lower costs. The *in silico* virtual screening method facilitates the development of innovative medicines. Based on the root mean square deviation results, the selected ligands, i.e., hydroxytyrosol and oleuropein, proved quite effective in inhibiting xanthine dehydrogenase (Fig. 5).

Xanthine oxidase results from the oxidation and/or proteolytic conversion of xanthine dehydrogenase. Xanthine oxidase is expressed in vascular cells and can circulate in plasma and bind to the extracellular matrix of endothelial cells. This enzyme catalyzes the metabolism of NADH, i.e., nicotinamide adenine dinucleotide (NAD) + hydrogen (H), molecular oxygen, hypoxanthine, and xanthine to produce O and HO. It is an important source of reactive oxygen species [54]. Most evidence about the involvement of xanthine oxidase in endothelial dysfunction and in the development of vascular diseases stems from the studies where the use of inhibitors of this enzyme (oxypurinol, allopurinol, febuxostat, topiroxostat) indicated improved endothelial function and vascular reactivity [29].

Recent studies showed that the inhibition of this enzyme protects against diabetic kidney disease and endometrial hyperplasia through the amelioration of oxidative stress by improving uterine-reduced glutathione and superoxide dismutase, as well as inhibiting the expressions of phosphatidylinositol-3-kinase (PI3K), Akt, and VEGF [54, 55]. Phenolic acids and flavonoids are antioxidants that can inhibit xanthine oxidase activity. The method was validated by attaching the inhibitory ligand to xanthine oxidase. Tran Minh *et al.* reported the root mean square deviation values ranging

from 1.019 to 2.35 [56]. Cinnamon phenolic acids showed a remarkable activity of xanthine dehydrogenase inhibition [57]. Similarly, Serrano *et al.* inhibited xanthine oxidase with cinnamic acid [58]. Mehmood *et al.* studied the xanthine oxidase inhibition mechanism of eight structurally diverse phenolic compounds commonly present in fruit plant (quercetin, quercetin-3-rhamnoside, 4,5-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, 3,4-O-di-caffeoylquinic acid, 4-O-caffeoylquinic acid, 3-O-caffeoylquinic acid, and caffeic acid) [59]. They used proton nuclear magnetic resonance (¹H NMR), atomic force microscopy, and various computational techniques. The study suggested that these phytochemicals had a potent inhibition and interaction with this enzyme. The inhibition of xanthine oxidase by phenolic compounds can be used to prevent several pathologies.

CONCLUSION

In the present study, *Olea europaea* L. leaves were rich in phenolic compounds, which possess potent antioxidant and antihyperlipidemic activities. *O. europaea* leaves improved the lipid profile in test animals by reducing such parameters as total cholesterol, low-density lipoprotein cholesterol, total triglycerides, total cholesterol vs. high-density lipoprotein cholesterol, and low vs. high density lipoprotein cholesterol. According to the histopathological tests, the liver of rabbits that received a hyper-lipid diet revealed changes in tissue architecture represented by steatosis deposits, bloating, and cellular necrosis. The histopathological architecture of liver sections obtained from rabbits treated with olive leaf powder demonstrated no damage in comparison with the group which received a high-fat diet only. Based on the energy and root mean square deviation, hydroxytyrosol was effective in inhibiting xanthine dehydrogenase. Thus, *O. europaea* leaves proved to contain a wide array of phytochemicals that could be used in the therapeutic context as an effective antihyperlipidemic agent and an inhibitor of xanthine dehydrogenase responsible for the production of free radicals. This potential can be used to prevent a number of diseases.

CONTRIBUTION

A. Amraoui and Z. Djerrou conceived and designed the analysis. A. Amraoui and S. Ali Haimoud collected the data. A. Amraoui, Z. Djerrou, K. Zerouki, and S. Elmokli worked with the data and analysis tools. A. Amraoui and Z. Djerrou performed the analysis. A. Amraoui and S. Ali Haimoud wrote the manuscript.

CONFLICT OF INTEREST

The authors declared no conflict of interests related to the publication of this article.

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