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# Antiatherogenic effect of *Pistacia lentiscus* via GSH restoration and downregulation of CD36 mRNA expression

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**Abstract**

*Pistacia lentiscus* var. Chia (Anacardiaceae) grows almost exclusively on Chios Island, Greece, and gives a resinous exudate resin used for culinary purposes by Mediterranean people. We investigated the molecular mechanisms through which total polar extract of the resin inhibits oxidized low-density lipoprotein (oxLDL) cytotoxic effect on peripheral blood mononuclear cell (PBMC). Cells exposed to oxLDL underwent apoptosis and necrosis, dependent on the duration of exposure. When culturing cells with oxLDL and the polar extract concurrently, we observed inhibition of both the phenomena. Because under oxidative stress the pro-oxidant systems outbalance the antioxidant, potentially producing oxidative damage and ultimately leading to cell death, we measured the levels of intracellular antioxidant glutathione (GSH). Additionally, we measured CD36 expression, a class B scavenger receptor, on CD14-positive cells, as CD36 has been identified as the oxLDL receptor in macrophages and may play a pivotal role in atherosclerotic foam cell formation. oxLDL decreased GSH levels and upregulated CD36 expression. *P. lentiscus* extract restored GSH levels and downregulated CD36 expression, even at the mRNA level. In order to find out the biologically drastic constituents of the resin's polar extract, fractions derived from RP-HPLC analysis were examined for their antioxidant effect on oxidatively stressed PBMC. The triterpenoid fraction revealed remarkable increase in intracellular GSH. We suggest GSH restoration and downregulation of CD36 mRNA expression as the pathways via which *P. lentiscus* triterpenes exert antioxidant/antiatherogenic effect. Additionally, our results provide strong evidence of the resin's antiatherogenic effect; therefore it is credited with beneficial health aspects.


*Keywords:* *P. lentiscus*; Phenolics; Triterpenes; oxLDL; PBMC; Apoptosis; GSH

## 1. Introduction

Evidence has accumulated that oxidized low-density lipoprotein (oxLDL) may play an important role in the initiation and progression of atherosclerotic lesions [1]. Under oxidative stress, free radicals attack plasma LDL that is oxidatively modified leading to the attraction of blood monocytes beneath the endothelium [2]. Monocytes differentiate into macrophages that themselves effect modifications in LDL, most importantly taking up the endothelial cell modified LDL [3]. The uptake of oxLDL occurs via scavenger receptors of the class SR-A and SR-B [4], a member of which is CD36 scavenger receptor [5] that binds to its lipid moiety [6]. Macrophages become susceptible to apoptosis. Nucleus shrinks; organelles change; membrane loses integrity; DNA breaks down. Eventually macrophages are converted to foam cells, full of cholesterol and oxidized lipids. Macrophage foam cells form the early atherosclerotic lesions [7] documented as the pathogenesis of cardiovascular heart disease.

In the search for compounds therapeutic against various diseases, several natural products have been investigated during the past few years, with promising prospects. Much attention has been focused on the potential antiatherogenic effects of components of the Mediterranean diet, such as red wine and olive oil. So far, in vitro and ex vivo experiments indicate that the adequate intake of phenolic compounds present in wine [8] and olive oil [9] is associated with a diminished risk of atherosclerosis. Apart from polyphenols, olive oil triterpenes have been proven as potent inhibitors of LDL oxidation in vitro [10]. Recently, the potent protective

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effect of the polar extract from *Pistacia lentiscus* resin, commonly known as Chios mastic gum, against the in vitro copper sulphate-induced LDL oxidation has been well documented [11]. Triterpenes present in *P. lentiscus* essential oil exhibit remarkable antioxidant effect on LDL [11]. *P. lentiscus* var. Chia grows almost exclusively in Chios Island (Greece) and gives resinous exudate resin after longitudinal incisions at close intervals from the base of the trunk up to the thicker branches of the tree. Chios mastic gum is basically consumed as chewing gum and also in other culinary art usage, especially in Greek, Turkish and Arabic kitchens, i.e. in the powder form as food additive, in the form of sugar containing gel as a sweetener and as mastic oil as a sweet additive in drinks. It has been referred to over centuries as having medicinal properties to treat a variety of diseases. It has been proven as a therapeutic agent against various gastric malfunctions, such as gastralgia, dyspepsia and gastric ulcer [12]. In 1999, Huwez et al. [13] published the potentiality of the resin to kill the bacteria *Helicobacter pylori*. In 2002, the hepatoprotective effect of the aqueous extract from the leaves of *P. lentiscus* on $CCl_4$ intoxicated rats was published [14]. To date though, no investigations have been carried out to highlight the activity of the resin on peripheral blood mononuclear cells (PBMCs) under oxidative stress. Monocytes are strongly implicated in atherogenesis [15] and are critical for T cell survival in the presence of oxLDL [16]. Thus, the aim of this study was to investigate the effect of total polar extract from *P. lentiscus* resin on the survival of oxLDL-treated PBMC. To determine this effect, we assayed PBMC to MTT and annexin V binding assays. Because DNA laddering is characteristic of programmed cell death, cell DNA was isolated and size-fractionated by gel electrophoresis. In an attempt to elucidate the mechanism, we measured glutathione (GSH) levels as it is an intracellular detoxification agent of toxic compounds and the oxLDL receptor CD36 expression, even at the transcriptional level. Finally, for the determination of the most drastic constituents of the resin's polar extract, fractions derived from RP-HPLC analysis were examined for their effect on oxidatively stressed PBMC.

## 2. Materials and methods

### 2.1. Materials

Sterilized LDL (lyophilized from 1 ml of LDL solution in 0.15 M NaCl and 0.01% EDTA at pH 7.4), phosphate buffer saline (PBS) tablets, Ficoll-Hypaque, acridine orange fluorescent dye (A-6014), ethidium bromide (E-8751) and MTT were purchased from Sigma Co. (St. Louis, MO, USA). The commercial LDL used in this study had been isolated from human plasma, as indicated by the manufacturer. Concerning its composition, labeled on the vial (L-5402) was the protein content (5.8 mg) and the cholesterol content (955 μg/mg protein). RPMI 1640 medium and fetal calf serum (FCS) were obtained from Gibco/BRL Life Technologies Inc. (Gaithersburg, MD, USA). Antibiotics (penicillin, streptomycin and L-glutamine) were obtained from Biochrom KG (Berlin, Germany). Monoclonal antibodies CD14-PE and CD36-FITC were from Serotec Inc. (Dosseldorph, Germany). Apoptosis detection kit and DNA laddering kit were purchased from R&D systems (Gaithersburg, MD, USA). Colorimetric assay for glutathione kit was obtained from OxisResearch Inc. (Portland, USA). RT-PCR reagents were all from Gibco. All other reagents were purchased from Sigma Co and chemicals from Aldrich Co. (Steinheim, Germany) and were of the highest purity available. *P. lentiscus* resin (Crude Chios mastic gum) was kindly donated by the Mastic Gum Growers' Association (Chios, Greece).

### 2.2. Extraction of P. lentiscus resin

*P. lentiscus* resin used in all experiments was crude normal, collected during spring 2003 and kindly donated from Chios Gum Mastic Growers' Association. All the resin given was extracted according to the solvent sequence methanol/water 60:40 (v/v) and methanol on the same day. In detail, it was subsequently extracted with the mixture (3 × 3 ml). The Folin Ciocalteau (FC) reaction was performed as has been previously described [17] and Folin Ciocalteau reactant substances were measured directly spectrophotometrically (UV-Vis 931 Uvicon Kontron Instrument) Because after the third extraction, FC reactant substances were not detectable, the resin was subjected to only three subsequent extractions. Next, the total resin from all three extractions was evaporated under a stream of nitrogen and the residue was dialyzed in methanol. FC reactant substances content from the total resin was 270 mg/kg. The methanolic solution was kept in a sealed cup at −8 °C until experiments. Before adjusting the volume to the desirable concentrations of the FC reactant substances and prior to its addition to cell culture, FC reactant substances were measured again and no significant differences were observed.

### 2.3. LDL modification

Commercial LDL was kept at −8 °C and a working suspension (200 μg protein/ml PBS) was prepared just before usage. The LDL oxidation test was performed as has been previously described [18] with slight modifications. In brief, 1.0 ml of working-LDL was mixed with 1.0 ml $CuSO_4$ (10 μM) as oxidizing agent and incubated at 37 °C for 6 h. The solution containing the resulted oxLDL was kept at −8 °C until use. To measure lipid peroxidation, three independent LDL oxidation tests were performed and afterwards the resulted suspensions were subjected to thiobarbituric acid (TBA) test. The TBA reactant substances (TBARS), mainly malonaldehyde, resulting from oxidation, were measured spectrophotometrically at 532 nm, after reaction with 2 ml of 1% alkaline TBA solution. TBARS were

evaluated using a standard curve of standard malonaldehyde in different concentrations versus absorption at 532 nm and were quantified at 2.69 (±0.34) μM malonaldehyde.

### 2.4. Cell culture

PBMC ($50–60 \times 10^6$) were isolated from healthy human volunteers as has been previously described [19]. Afterwards, they were resuspended in complete medium consisting of RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin/streptomycin. PBMC were added to each well of a 24-well plate at a density of $2 \times 10^6$ cells/ml and at final volume 2 ml, either in medium only (control), or with oxLDL (the solution of which had just before been evaporated under a stream of nitrogen and resuspended in 1 ml of the above-described complete medium), or with oxLDL (treated as above) and concentrated polar extract from *P. lentiscus* resin of known FC reactant substances. Individual 2 μl extract volumes containing 5.4, 54 and 540 μg of FC reactant substances (equivalent to caffeic acid) were added in individual cell culture of 2 ml medium, resulting in the desirable concentration of FC reactant substances (that is 2.7, 27 and 270 μg/$2 \times 10^6$ cells/ml medium respectively). Dilution of methanol to cell medium was chosen 1/1000 (v/v) in order to eliminate the effect of methanol. Cells were cultured in a humidified cell incubator under 5% $CO_2$ and 37 °C. At the end of each incubation period, cells were harvested and subjected to different assays.

### 2.5. Cell viability assay

Effect on cellular viability was evaluated via MTT assay as has been previously described [20]. The optical density of the cellular homogenate was measured at 550 nm using an Elisa reader (Versamax). As a background value, a well containing only complete medium plus MTT plus isopropanol was used. Each experiment was carried out in triplicate. Fractional absorbance was calculated using the equation:

$$\frac{\text{mean absorbance in three test wells} - \text{absorbance in background well}}{\text{mean absorbance in three control wells} - \text{absorbance in background well}} \times 100.$$

### 2.6. Annexin V binding assay

Percentages of apoptotic and necrotic cells were assayed by annexin V-FITC and propidium iodide (PI)-staining on a Becton & Dickinson FACSscan. Measurements were performed using the apoptosis detection kit as recommended by the manufacturer. Each experiment was carried out in triplicate.

### 2.7. Fluorescence microscopy

To distinguish living from apoptotic and dead cells, PBMC, of the same cell cultures as those disposed to annexin V binding assay, were stained with acridine orange fluorescent dye (100 μg/ml) mixed with ethidium bromide (100 μg/ml) in PBS–bovine serum albumin (1%) and were immediately examined under a fluorescence microscope (Leica, DC 300F).

### 2.8. Analysis of DNA fragmentation

For the analysis of DNA laddering, characteristic of programmed cell death, DNA was isolated from $2 \times 10^6$ cells using a DNA laddering kit as indicated by the manufacturer.

### 2.9. Flow cytometric analysis of CD36

At the end of incubation, $10^5$ cells were washed with PBS and afterwards doubled stained with PE-labeled CD14 and FITC-labeled CD36 for 30 min at 4 °C. The cells were then washed twice with PBS to remove the bound antibodies and were immediately analyzed on a FACSscan. Monocyte population was gated and percentage of CD36 on CD14-positive cells was measured using CellQuest software. Each experiment was carried out in triplicate.

### 2.10. Assay for GSH

At the end of incubation, cells were extracted with 5% metaphosphoric acid solution and then centrifuged at 2000 × *g* for 10 min. The resulting supernatant was separated and the GSH assay was performed using the colorimetric assay for glutathione as indicated by the manufacturer. GSH concentration was evaluated using a standard curve of absorbance units versus GSH concentrations and expressed as μM. Each experiment was carried out in triplicate.

### 2.11. Quantification of mRNA expression

Total RNA was extracted using a phenol/guanidine isothiocyanate method according to the manufacturers' instruction. Briefly, PBMC were precipitated and lyzed in 1 ml of Trizol™ reagent and stored at −70 °C until used. One half of the lysate was then subjected to one round of chloroform extraction, isopropanol precipitation, 70% ethanol wash, and the RNA was then vacuum dried and resuspended in 10 μl RNase-free water. Reverse transcription was performed at 37 °C in a 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$ buffer containing 10 mM DTT, 0.4 mM dNTPs, 0.5 μg random hexamer primers and 200 units of reverse transcriptase (Superscript™, Invitrogen). cDNA was stored at –20 °C until used as template for semiquantitative RT-PCR. To that end, the cDNA was PCR amplified in the presence of 2.5 mM $MgCl_2$, 400 μM dNTPs, 2.5 units Taq polymerase and 0.25 μM of the following primer pairs:

CD36L: 5′-CAGCCCAATGGAGCCATC-3′ and CD36R: 5′-CAGCGTAGATAGACCTGC-3′, amplifying a 487 bp fragment of the CD36 gene [21] and actin-sense: 5′-TGACGGGGTCATCCACACTGTGCCCATCTA-3′ and actin-antisense: 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′, amplifying a 661 bp fragment of the β-actin gene. Conditions used were 35 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 40 s for CD36 and 30 cycles of 94 °C for 40 s, 50 °C for 40 s and 72 °C for 1 min for β-actin amplification. Subsequently, 20% of the PCR reaction volume was run in a 1.8% agarose gel. Band densities were measured and analyzed with the BandLeader™ V3.00 software. The ratios of CD36 to β-actin densities are considered to reflect relative CD36 mRNA abundancy. Each experiment was carried out in triplicate.

### 2.12. HPLC analysis

Isolation of the resin polar extract fractions was accomplished by RP-HPLC. An HPLC system (Agilent Technologies, model HP 1050, Waldbronn, Germany) combined with auto-sampler, diode array detector (HP-1050), fluorescence detector (HP 1046A) and data software was used. Analysis of *P. lentiscus* extract and isolation of fractions was performed as previously described [22] slightly modified for the present study [23]. Briefly, a quaternary solvent system was used consisting of water, methanol, acetonitrile and isopropanol, with gradient elution on a Nucleosil C18100-7 (125 mm × 8 mm) column (Macherey-Nagel, Düren, Germany). Injections of 100 μl from a solution of *P. lentiscus* extract in methanol were performed.

### 2.13. GC–MS analysis

Fractions from HPLC were evaporated under a stream of nitrogen. Trimethylsilyl derivatives were prepared after reaction of dry residues with BSTFA and TMCS at 70 °C for 30 min. GC–MS data were obtained with a fused silica 5% phenyl–95% methyl siloxane column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), in a gas chromatograph (HP 6890) coupled to the ion source of a MSD (HP 5972). Helium was used as carrier gas with a linear velocity of 27 cm/s. The temperature was programmed at 70 °C for 5 min, then ramped at the rate 15 °C/min to 130 °C and subsequently at rate 4 °C/min to 170 °C where it remained for 15 min. Finally, temperature was ramped to 300 °C at a rate of 10 °C/min and remained to this final point for 30 min. The mass spectrometer was scanned from *m/z* 35 to 700 amu and ions were generated by EI (70 eV). MS information was interpreted and compared to Nist Mass Spectral Library.

### 2.14. Evaluation of the biologically drastic compounds

To find out whether the effect was due to the polyphenolic content or due to the triterpenoid content of the resin, cells were cultured under the above-mentioned culture conditions with oxLDL and each of the fractions derived from HPLC. After the end of incubation, the supernatants collected were subjected to GSH assay.

### 2.15. Statistical analysis

Data were expressed as means ± S.D. Comparisons were determined using the paired *t*-test. Differences between the means were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Effect of P. lentiscus resin extract on oxLDL-induced cytotoxicity

Cytotoxicity of oxLDL on PBMC and resistance when exposed to the extract were assayed via MTT assay. As shown in Fig. 1, for 48 h in culture, oxLDL revealed cytotoxic effect on PBMC resulting in significant decrease in their number to 60.1 ± 2.0% living cells. Fig. 1 also leads us to conclude that addition of 2.7 μg/ml of the extract inhibits cytotoxicity to some extent (63.2 ± 3.0% survival), while 270 μg/ml medium of the polar extract inhibits cytotoxicity extensively (95 ± 3.2% survival). Ten-fold decrease to 27 μg/ml medium exhibits similar protection to revealed at the higher concentration revealed (90.0 ± 2.4% survival). Therefore, concentration equivalent to 27 μg/ml of the extract was used in all subsequent experiments. Fig. 2 indicates that when exposing PBMC to oxLDL for 48 and 72 h, the minimum and maximum lengths of time selected respectively, living cells decreased to 64.5 ± 2.7% and 66.1 ± 3.1% in that order. In the presence of 27 μg/ml, cytotoxicity was inhibited almost totally. Inhibitory effect of the extract on cytotoxicity was in all cases statistically significant.

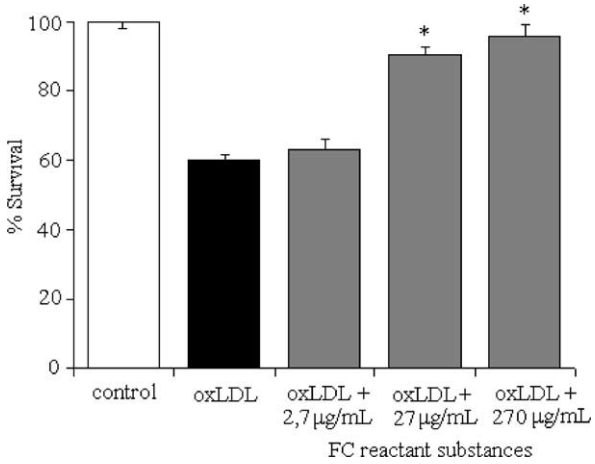


Fig. 1. Effect of different concentrations of Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL) cytotoxicity against PBMC. Cell viability was assessed via the MTT assay. Values are means ± S.D. of three independent experiments. Asterisk (∗) points out statistically ($P < 0.05$) significant results.

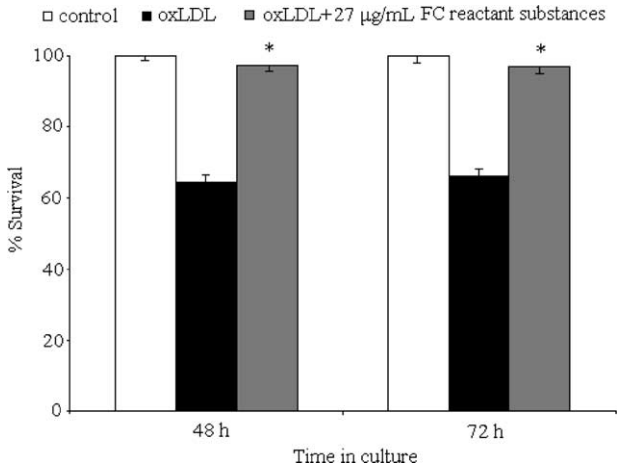


Fig. 2. Time-dependent effect of 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL) cytotoxicity against PBMC. Cell viability was assessed via MTT assay. Values are means ± S.D. of three independent experiments. Asterisk (∗) points out statistically ($p < 0.05$) significant results.

### 3.2. Inhibition of oxLDL mediated apoptosis and necrosis by P. lentiscus resin extract

To examine the effect of *P. lentiscus* extract on survival of PBMC, we assayed cultured cells for the ability to undergo apoptosis or necrosis. Annexin exhibits selective affinity for negatively charged phospholipids of the apoptotic cell membranes, while PI stains necrotic cells with disruptive internal and external membranes. Under salt and calcium concentrations, annexin V is predisposed to bind phosphatidylserine over most other phospholipids species present in many sites on the cell surface, therefore resulting in a very intense signal. In Fig. 3, flow cytometric analysis of annexin V and PI-labeled PBMC indicated that cells exposed to neither oxLDL nor polar extract for 48 h were 99.6% living (Fig. 3A), whereas when exposed to oxLDL for 48 h were 9.36% apoptotic and 4.58% necrotic (Fig. 3B). On the other hand, PBMC cultured under oxidative and extract conditions for 48 h simultaneously were 0.55% positive for annexin and 2.87% positive for PI (Fig. 3C). In Fig. 4 where PBMC cultured for 72 h are presented, under no agent 99.2% were alive (Fig. 4A), while under oxLDL only 0.82% underwent apoptosis and 73.5% underwent necrosis (Fig. 4B). Inhibition of the phenomenon of necrosis to 4.04% cells was detected under oxidative and extract conditions (Fig. 4C), indicating the antioxidant effect of *P. lentiscus* polar extract. We also checked oxLDL and polar extract effect on PBMC by analysis of DNA fragmentation. As shown in Fig. 5, the classical DNA ladder assay shown by gel electrophoresis did not reveal any apoptotic pattern

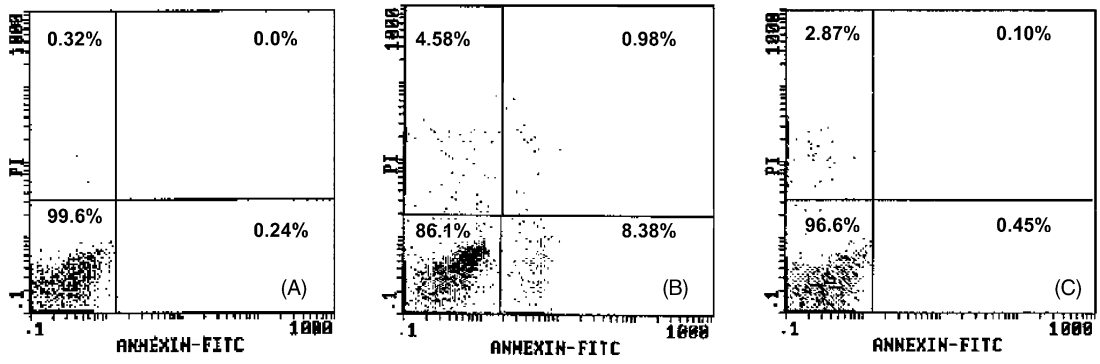


Fig. 3. FACS analysis of annexin V and PI binding of PBMC cultured for 48 h. (A) PBMC cultured in the absence in medium only (control); (B) PBMC cultured with oxidized LDL (oxLDL); (C) PBMC cultured with both oxLDL and 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.). Data are representative of three independent experiments.

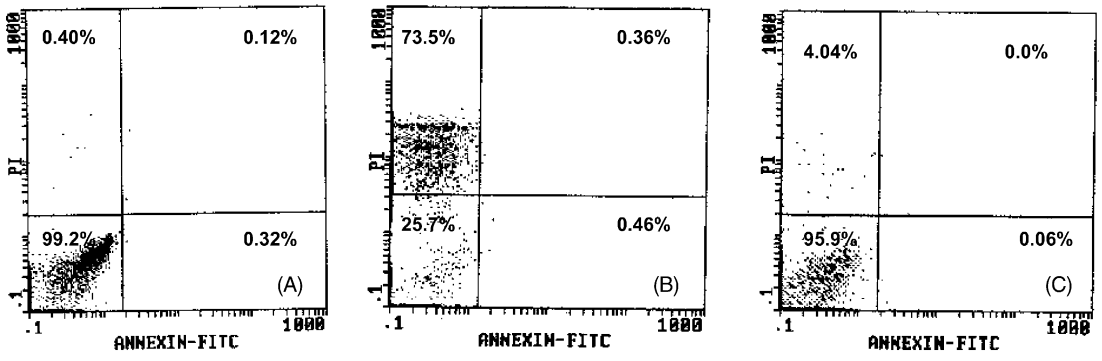


Fig. 4. FACS analysis of annexin V and PI binding of PBMC cultured for 72 h. (A) PBMC cultured in the absence of oxidized LDL (oxLDL) or polar extract (control); (B) PBMC cultured with oxLDL; (C) PBMC cultured with both oxLDL and 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.). Data are representative of three independent experiments.

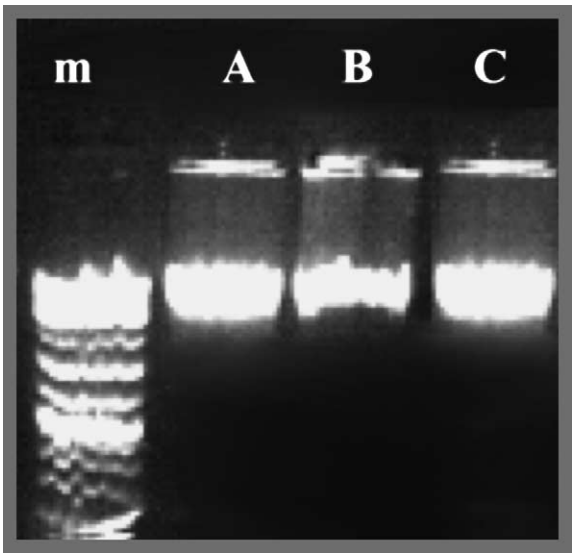


Fig. 5. DNA fragmentation analysis of PBMC cultured for 48 h in medium only (lane A), with oxidized LDL (oxLDL) (lane B), with oxLDL and 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) (lane C). The classical DNA ladder assay shown by gel electrophoresis did not reveal any apoptotic pattern in oxLDL-treated PBMC. Lane m: marker.

in oxLDL-treated PBMC. However, the absence of DNA degradation is perhaps due to a progressive loss of cleaved DNA from cells. DNA electrophoreses analysis may not determine whether cells undergo apoptosis since it does not lend itself to rapid multiparameter analysis at the single cell level. DNA laddering is a late apoptotic event. In the present study, the number of late apoptotic cells (0.98 out of 9.36%, Fig. 3) is too low to justify a DNA laddering pattern, while early apoptotic cells potentially do not present a DNA laddering pattern. Fluorescence microscopy, a non-quantitative assay of analysis, relies dependability upon dyes that stain either viable or non-viable cells. To observe microscopically the effect of *P. lentiscus* polar extract on PBMC under oxidative stress, cells were stained with acridine orange and ethidium bromide. Both dyes intercalate into DNA and stain nuclei green and orange, respectively. Viable cells have intact nuclei and stain green. Apoptotic cells have very visible piknotic nuclei with DNA condensation and stain yellow. Necrotic cells fluoresce orange. Fig. 6 indicates that in the absence of both oxLDL and the polar extract (control), PBMC were viable after 48 h in culture (Fig. 6A), even after the extended 72 h length of time (Fig. 6D). Induced oxidative conditions triggered apoptosis when culture lasted 48 h (Fig. 6B), whereas 72 h incubation with oxLDL led to extensive necrosis (Fig. 6E). Concurrent presence of the polar extract inhibited apoptosis (Fig. 6C) and necrosis (Fig. 6F).

### 3.3. CD36 expression

CD36 of SR class B has been identified as the oxLDL receptor. When oxLDL bind to CD36 scavenger receptors on the external membrane of macrophages that have migrated to the inner layer of the aorta they inhibit their reversion to blood. Thus, macrophages enhance the formation of foamed cells and the cytotoxic effect on the endothelium that leads to loss of its integrity. Previous research has indicated enhanced expression of CD36 on oxLDL-treated macrophages [21]. As shown in Fig. 7, when gating on oxLDL-treated monocytes/macrophages, flow cytometric analysis indicated 5.3% rise of CD36 expression for 48 h in culture, while for 72 h in culture 6.4% rise. Statistically significant decrease was observed when treating cells with oxLDL and polar extract concurrently. That is, from $45.3 \pm 1.8\%$ to $39.6 \pm 3.0\%$ CD36 expression for 48 h in culture and from $50.3 \pm 2.4\%$ to $19.3 \pm 3.2\%$ for 72 h in culture.

### 3.4. GSH levels

GSH plays a pivotal role in the defense against oxidative insult. Our findings, given in Fig. 8, indicate that treatment of PBMC with the oxidized form of LDL resulted in decrease of GSH levels. PBMC treated with oxLDL for 48 h revealed 20.0% decrease of GSH, similar to 21.4% decrease when treating PBMC with the oxidizing agent for 72 h. *P. lentiscus* extract restored GSH levels utterly when treating cells under oxidative stress.

### 3.5. CD36 mRNA expression

Results of CD36 mRNA expression are given in Figs. 9 and 10. Control populations expressed CD36 at the mRNA level (Fig. 9, lanes A and B) that was upregulated in the presence of oxLDL (Fig. 9, lanes C and D). Extra bands in lanes C and D are perhaps due to non-specific PCR amplification of similar to CD36 molecules after the oxLDL induction. Incubation of cells with oxLDL and the polar extract concomitantly, released significant decrease in mRNA expression (Fig. 9, lanes E and F). That is, after 48 h in culture, oxLDL raised CD36 mRNA expression from 35 to 75%, while simultaneous presence of total polar extract from *P. lentiscus* constrained CD36 mRNA expression to 8% (Fig. 10). Similarly, after 72 h in culture, presence of oxLDL increased message for CD36 from 81 to 100%, whereas polar extract inhibited CD36 expression at the mRNA level to 6% (Fig. 10).

### 3.6. Biologically drastic compounds

Based on both the HPLC and GC chromatographic profile of polyphenolic and triterpenoid standards, the first HPLC fraction obtained was considered to contain the resin polyphenols and the second the triterpenoids. For the purpose of verification and characterization of each fraction, GC–MS analysis was performed [23]. Measurement of GSH levels after PBMC treatment individually with the polyphenolic and triterpenoid HPLC fractions revealed an increase in GSH 5.0% when treating cells with oxLDL and

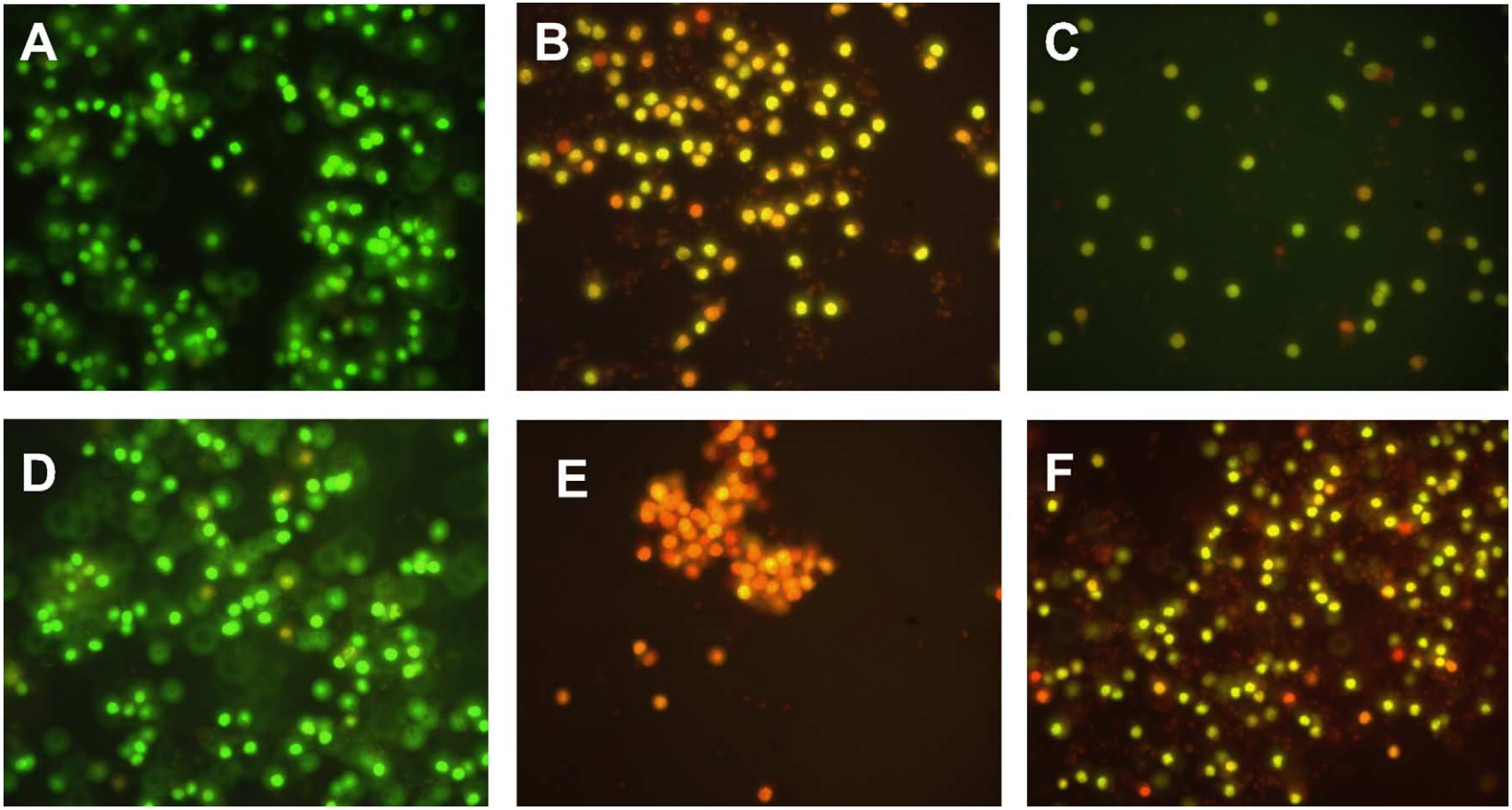


Fig. 6. Fluorescence microscopy of acridine orange- and ethidium bromide-stained PBMC. PBMC were cultured for 48 h (A–C) and 72 h (D, E, F) in the absence of any agent (A, D), with oxidized LDL (oxLDL) (B, E) and with oxLDL and 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) (C, F).

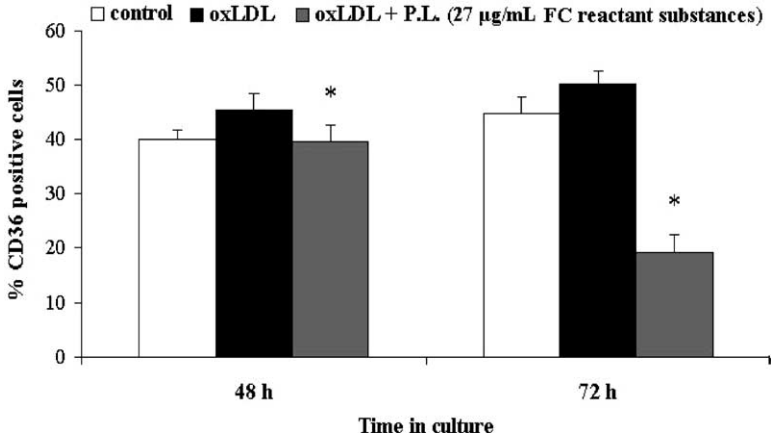


Fig. 7. Time course effect of the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced CD36 expression. PBMC doubled stained with PE-labeled CD14 and FITC-labeled CD36. Monocyte population was gated and percentage of CD36 on CD14-positive cells was measured using CellQuest software. Values are means ± S.D. of three independent experiments. Asterisk (∗) points out statistically ($P < 0.05$) significant results.

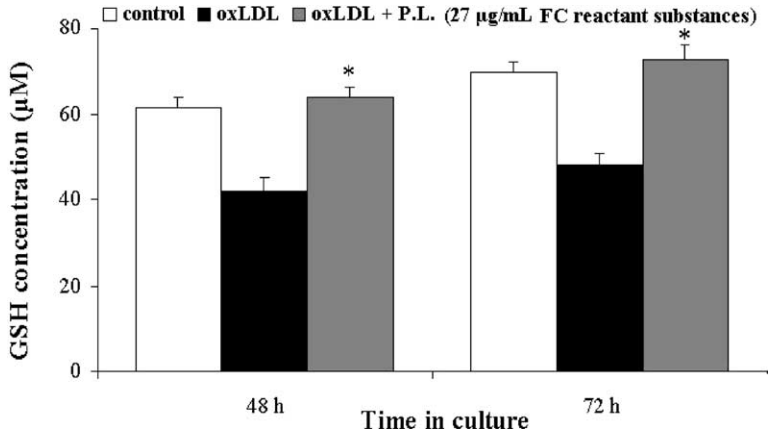


Fig. 8. Time course effect of 27 μg/ml Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced glutathione (GSH) depletion. Asterisk (∗) points out statistically ($P < 0.05$) significant results.

the polyphenolic fraction and 23.6% when treating cells with oxLDL and the triterpenoid fraction, compared to GSH measured when culturing cells with oxLDL alone (Fig. 11).

## 4. Discussion

In the present paper, it is demonstrated for the first time that enrichment of oxLDL cultured PBMC with the polar extract from *P. lentiscus* is associated with diminished cell mortality. Evidence of the antiatherogenic effects of several antioxidants, including polyphenols or triterpenes, on crucial cells implicated in atherogenesis, such as endothelial cells, platelets, smooth muscle cells and macrophages, is nowadays mounting. Among other constantly shown antioxidant properties of polyphenols and triterpenes, most in vitro studies indicate protective effect against LDL oxidation [10,11,24]. Apart from the aforementioned in vitro antioxidant effect of the polar extract from *P. lentiscus* resin [11], it has also been shown that the polar saliva-extract of the resin obtained when chewing it as a gum exhibits protective effect against LDL oxidation as well [25]. Consistently with these findings, investigation of the effect of the resin's polar extract on PBMC response to oxLDL is important, as it sheds light on its possible antiatherogenic properties.

Hereby, it is shown, by different assays, that oxLDL is highly cytotoxic on total PBMC population. Because we used copper sulphate (5 μM final concentration) to modify LDL to its oxidized form, PBMC were also cultured with copper sulphate in the same final concentration. Neither MTT, nor annexin V binding assay showed any effect of copper sulphate on cells (data not shown), thus eliminating any possibility that copper sulphate interfered to cytotoxicity itself. Our findings point out that oxLDL owes cytotoxicity to decrease in GSH. This is in accordance with the recently published results [26] showing that oxLDL is primarily responsible for GSH depletion creating an oxidizing environment required for γ-GSC induction and compensatory GSH synthesis. Ghibelli et al. [27] have shown that GSH decrease occurs during cell death through a physiological process, i.e.

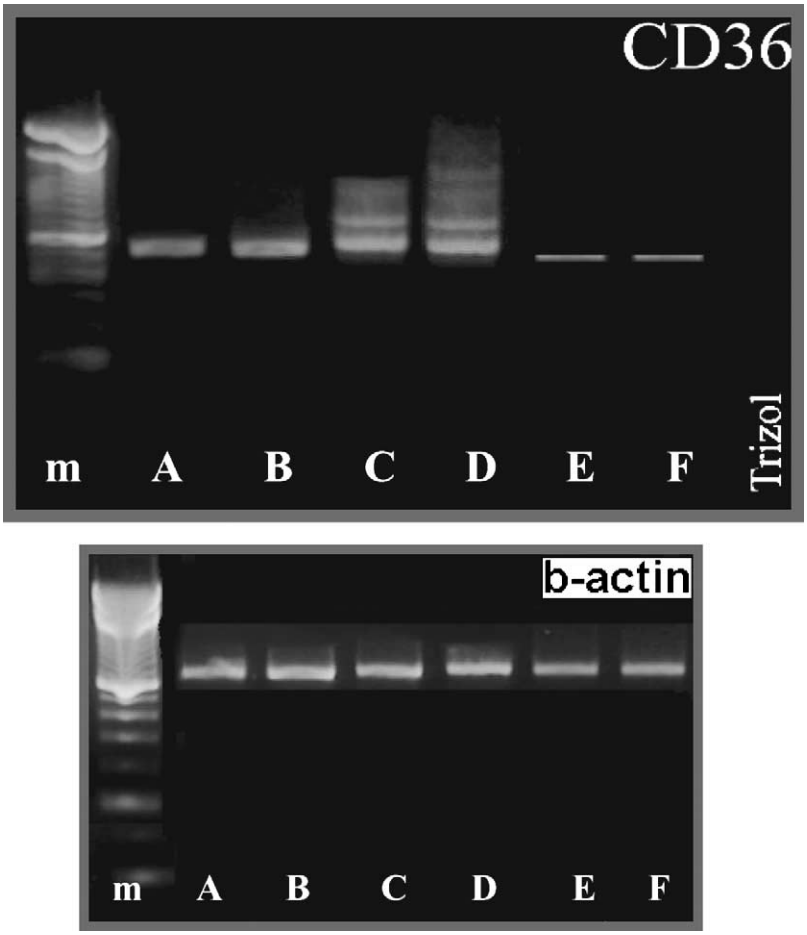


Fig. 9. Effect of the polar extract (27 μg/ml) from *P. lentiscus* resin enrichment of cells on CD36 mRNA. CD36 mRNA was quantified by RT-PCR, using β-actin as control. PBMC cultured in the absence of oxidized LDL (oxLDL) and Folin Ciocalteau (FC) reactant substances in the polar extract from *P. lentiscus* resin (P.L.) for 48 h (lane A) and for 72 h (lane B); PBMC cultured with oxLDL for 48 h (lane C) and for 72 h (lane D); PBMC cultured with oxLDL and FC reactant substances in the polar extract from P.L. resin for 48h (lane E) and for 72 h (lane F). Lane m: marker.

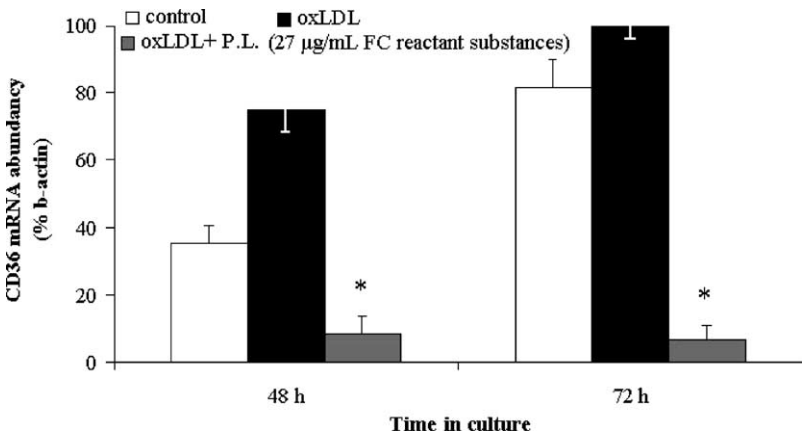


Fig. 10. Time course effect of the polar extract [27 μg/ml Folin Ciocalteau (FC) reactant substances] from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced CD36 mRNA expression. Values are means ± S.D. of three independent experiments. Asterisk (∗) points out statistically ($P < 0.05$) significant results.

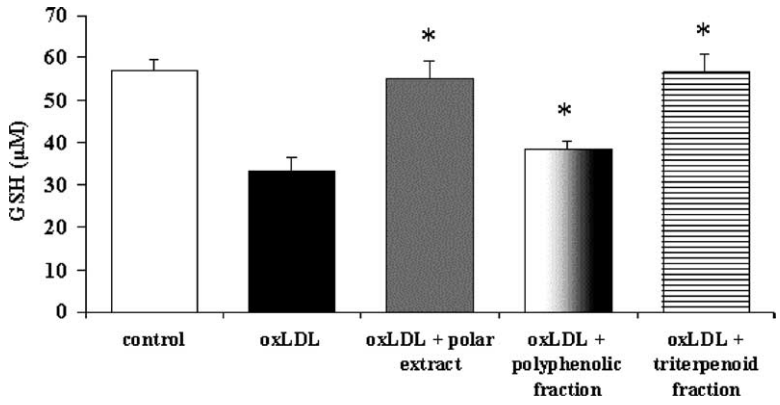


Fig. 11. Effect of the triterpenoid and polyphenolic fraction of the polar extract from *P. lentiscus* resin (P.L.) on oxidized LDL (oxLDL)-induced glutathione (GSH) depletion. Values are means ± S.D. of three independent experiments. Asterisk (∗) points out statistically ($P < 0.05$) significant results.

via physiological carriers responsible for GSH efflux. When inhibiting the carriers, they not only observed GSH restoration, but also reduced cell death, thus proving that decrease in GSH is an intrinsic part of cell death signaling, a necessary step to trigger the events of apoptosis. Here, it is shown that oxLDL induces cytotoxicity on PBMC via GSH depletion.

Our findings suggest that PBMC undergo severe damage when subjected to oxLDL. In accordance with previous work [28], it is observed that cellular responses to oxLDL were dependent on the duration of exposure. That is, apoptosis for 48 h exposure and severe necrosis for 72 h exposure. As far as monocytes are concerned, loading with oxLDL significantly increased CD36 expression. Feng et al. [29] reported that monocyte CD36 expression is upregulated in the presence of oxLDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ. Devaraj et al. [21] observed that CD36 expression is upregulated indeed, however after 96 h in culture, and gets maximal (five-fold) after 8–10 days. We show that 5% increment in CD36 expression occurs after 72 h in culture, indicative of ongoing monocyte-to-macrophage differentiation. It was also examined whether the expression of CD36 was due to respective message for CD36. Compliant with that study, we show that CD36 mRNA expression was upregulated in the presence of oxLDL, meaning that expression increases even at the transcriptional level. The significance of enhanced CD36 expression is due to the fact that once macrophages attract oxLDL to scavenger receptors, they promote endocytosis and convert into cholesterol-loaded foam cells. In our study, the reduced uptake of oxLDL might be attributed to the downregulation of CD36 in the presence of the extract, both at the protein and the mRNA levels.

In conflict with the above-mentioned oxLDL effects, the polar extract from *P. lentiscus* resin was proven to inhibit cytotoxicity, both apoptosis and necrosis, restore GSH levels, downregulate CD36 expression even at the transcriptional level. To clarify whether the extract's effect was on oxLDL or cells, PBMC were subjected to (a) oxLDL for 24 h and (b) oxLDL for 24 h, cells were washed to collect the supernatant and then cultured for another 24 h with the polar extract. At the end of incubation, GSH assay was performed in both cultures revealing 22.0% decrease under oxidative conditions compared to the control, but total restoration when removing oxLDL and adding the polar extract (data not shown). The result drove us to conclude that the extract is drastic on PBMC rather on oxLDL.

Interestingly enough, when comparing the FC reactant content in extract from *P. lentiscus* of different time collection, different values are obtained (data unpublished, manuscript in preparation). This observation led us to use the extract of a certain portion of 2003 springtime collection. The identification and quantification of polyphenols and triterpenes in the polar extract of the resin has been recently published by our team [23], while triterpenes have been broadly investigated in the past by Papageorgiou et al. [30]. Among the constituents remained in the polar extract, triterpenes were the most active on the antioxidant defence of PBMC. Mass spectra indicated oleanolic acid and urs-12-en-28-al as the major components of the triterpenoid fraction (data not shown) while indicated in the polyphenolic fraction were tyrosol, *p*-hydroxy-benzoic acid, *p*-hydroxy-phenylacetic acid, vanillic acid and traces of gallic and *trans*-cinnamic acids. Oleanolic acid and isomer ursolic acid have been found to increase glutathione and superoxide dismutase in Dahl salt-sensitive insulin resistant rat model of genetic hypertension [31]. They have been also proven to exhibit antioxidant effect [11], antihyperlipidemic and antihypertensive effects and to prevent the development of atherosclerosis [32]. Regarding polyphenols, these have been proven to be potent antioxidants [33].

Bearing all in mind, the resin enhances cell defense against oxidative stress via pivotal physiological pathways, i.e. GSH and CD36, rather than counteracts oxLDL. Up to now, little did we know about its biological properties. However, this study is the first to indicate a new potential therapeutical use, beyond the antiulcer one, of this species, owed mainly to its triterpenoid content. *P. lentiscus* resin is indicated as a novel antioxidant/antiatherogenic agent of the Mediterranean areas.

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