



Biological effects and potential mechanisms of action of *Pistacia lentiscus* Chios mastic extract in Caco-2 cell model



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ABSTRACT

Pistacia lentiscus L. (PL) is an evergreen shrub from which it is derived a precious aromatic resin called mastic gum or mastic. This extract possesses numerous properties, such as antimicrobial, anti-inflammatory, anticancer, etc. The aim of this study was to investigate the biological activities of a patented PL Chios mastic extract in a human small intestine mucosa model, the Caco-2 cells. PL Chios mastic extract showed no toxic effect in Caco-2 cells treated with lower concentrations (< 100 µg/ml). Pro-inflammatory cytokines were not increased in Caco-2 cells and disaccharidase activity as well as sucrase–isomaltase expression were decreased at 50 µg/ml, suggesting a potential role in glycaemic control. Also paracellular permeability was reduced in Caco-2 cells and remarkably this extract induced a barrier effect useful in protecting against chemical or biological insults.

1. Introduction

Pistacia lentiscus L. (PL) is an evergreen shrub of the family *Anacardiaceae* commonly present in the Mediterranean area (Conti, Abbate, & Alessandrini, 2005), especially in the Greek island of Chios where it is cultivated for its fragrant and aromatic resin. This resin is called *mastic gum* or *mastic* (known as the “tears of Chios”) an exudate obtained from the stem and leaves of PL var. *Chia*. This preparation is traditionally used as food additive and flavouring agent, and can be found in Greek cakes, liqueurs and many dishes (Paraschos, Mitakou, & Skaltsounis, 2012). Moreover, PL mastic is present in the production of perfumes, fragrances, lotions and toothpaste. In ethnomedicine, PL mastic has been used for more than 2500 years in Mediterranean basin to treat gastrointestinal diseases, such as gastralgia, peptic ulcer, dyspepsia, diarrhoea, stomach upsets, nausea (Dimas, Pantazis, & Ramanujam, 2012). More recently PL has been reported to possess antimicrobial, anti-inflammatory, antioxidative, wound-healing, neuroprotective, antidiarrheal, anti-atherosclerotic, hypotensive, hypoglycemic, diuretic, anti-urolithiasis and anticancer effects (Benhammou, Bekkara, & Panovska, 2008; Dimas et al., 2012; Ljubuncic, Song, Cogan,

Azaizeh, & Bomzon, 2005; Paraschos et al., 2012; Rauf et al., 2017; Yemmen, Landolsi, Ben Hamida, Megraud, & Trabelsi Ayadi, 2017). Numerous preclinical works have been published that corroborate pharmacological activities of PL mastic, nonetheless a small number of studies involving humans have been published. Two studies showed a decrease in glucose and cholesterol levels (and other biochemical serum parameters) by administration of Chios mastic (Kartalis et al., 2016; Triantafyllou, Chaviaras, Sergeantanis, Protopapa, & Tsaknis, 2007). Furthermore a double-blind placebo controlled trial showed that Chios mastic ameliorated symptoms in dyspeptic patients (Dabos et al., 2010). Similarly a pilot study reported bactericidal activity of Chios mastic in patients harbouring *Helicobacter pylori* infection (Dabos, Sfika, Vlatta, & Giannikopoulos, 2010) and another pilot study showed beneficial effects on Crohn’s disease (Kaliora, Stathopoulou, Triantafyllidis, Dedoussis, & Andrikopoulos, 2007).

Given PL mastic interesting properties reported in literature, we decided to investigate its effects at cellular level in a colon cell model that can resemble morphologically and functionally, under specific conditions, the biology of enterocytes of the human small intestine mucosa (Sambuy et al., 2005). In particular, we decided to adopt Caco-

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2 cells because they are the most used and investigated model to study human absorption and transport processes (Delie & Rubas, 1997; Jochems, Garssen, van Keulen, Masereeuw, & Jeurink, 2018). A standardized preparation of PL mastic rich in triterpenes was analysed and the properties of this extract was explored evaluating cell viability, cell clonogenic ability, cell morphology, cell cycle distribution, pro-inflammatory cytokines, disaccharidase activity, gene expression of sucrase–isomaltase complex, paracellular permeability assay and wound healing assay.

2. Materials and methods

2.1. Materials and reagents

All reagents and solvents were purchased from Sigma-Aldrich (Milano, Italy), if not otherwise indicated. DMEM-F12 + GlutaMAX, FBS, 0.05% trypsin-EDTA and antibiotics were from Thermo Fisher Scientific (Milano, Italy). DMSO (dimethyl sulfoxide) was used to dissolve the *Pistacia lentiscus* Chios mastic and its concentration never exceeded 0.1% in every experiments.

2.2. *Pistacia lentiscus* extract (ES)

The *Pistacia lentiscus* Chios mastic extract was labelled as ES. This abbreviation was used through all the text. This extract (ES) was kindly provided by Nova Salus Fitolab (Via Galvani, 12, 35030 Rubano PD, Italy). ES was obtained from a patented extraction procedure that permits to have a raw material named “Supermastic” with very high contents of triterpenes and elimination of polyterpene polymers. This extract was further analytically characterized and used in this study to determine its biological properties.

ES was characterized by means of HPLC-ESI-MS (Fig. 1) and main terpenes species (Table 1) were detected by specific reference standards if commercially available or by their fragments produced by tandem mass and MSⁿ if necessary. Due to the lack of all standards for identified terpenic species, quantification of total extracted terpenes was performed by using oleanolic acid (Aldrich, > 97%) as unique reference standard. The total content of terpenic acid in ES was found to be 90–95% w/w.

2.3. Cell culture

Caco-2 cells were kindly provided by Professor N. Ferri, Department of Pharmaceutical and Pharmacological Sciences, University of Padova (Italy). Caco-2 (adenoCarcinoma of the COlon) cells are human

Table 1

Main terpenic species identified in the Supermastic ES.

$(m/z)^{-1}$	Terpenic species
453 C ₃₀ H ₄₆ O ₃	Oleanonic acid (peak 42) Moronic acid (peak 44) 24Z-isomasticadienonic acid (peak 53) 24Z-masticadienonic acid (peak 52)
455 C ₃₀ H ₄₈ O ₃	Oleanolic acid (peak 41) 24Z-masticadienonic acid (peak 47) 24Z-isomasticadienonic acid (peak 54)
469 C ₃₀ H ₄₆ O ₄	Enoxolone acid (peak 20) Albasapogenin acid (peak 32) Coleonolic acid (peak 37) Glycyrrhetic acid (peak 46)
471 C ₃₀ H ₄₆ O ₃	Hederagenic acid (peak 50) Maslinic acid (peak 51) Corosolic acid (peak 43)
501 C ₃₀ H ₄₆ O ₆	Medicagenic acid (peak 7) Idrossioxopomolic acid (peak 8)

adenocarcinoma cells first isolated in the 1970s that can express differentiation features characteristic typical of enterocytes. Cells were grown in DMEM/F-12 + GlutaMAX supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin 10,000 U/ml. Cell cultures were maintained in incubator at 37 °C with 5% CO₂ and sub-cultured at 70–80% confluence.

2.4. MTT cell viability assay

Cell viability was analysed by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay as previously described (Pezzani et al., 2016). Briefly, Caco-2 cells (1.0 × 10⁴ cells/well in 96-well plates) were grown for 48 h and then treated with ES 1, 10, 50, 100, 500 or 1000 µg/ml. After 1, 3, 6 or 24 h of incubation, water-soluble MTT was added to each well and cells were incubated at 37 °C for 3 h. Formazan salts were solubilized by DMSO and absorbance at 550 nm was measured with a VICTOR X3 Plate Reader (PerkinElmer, Milano, Italy). Background absorbance at 620 nm was also measured and subtracted from the 550 nm measurement. The results were expressed as a percentage of untreated control (100%). Each analysis was performed in triplicate and repeated 3 times.

2.5. Clonogenic assay

Caco-2 cells were seeded in 6-well plates at a low density (1000 cells per well) and treated with ES 50, 100, 250 and 500 µg/ml for 24 h (and

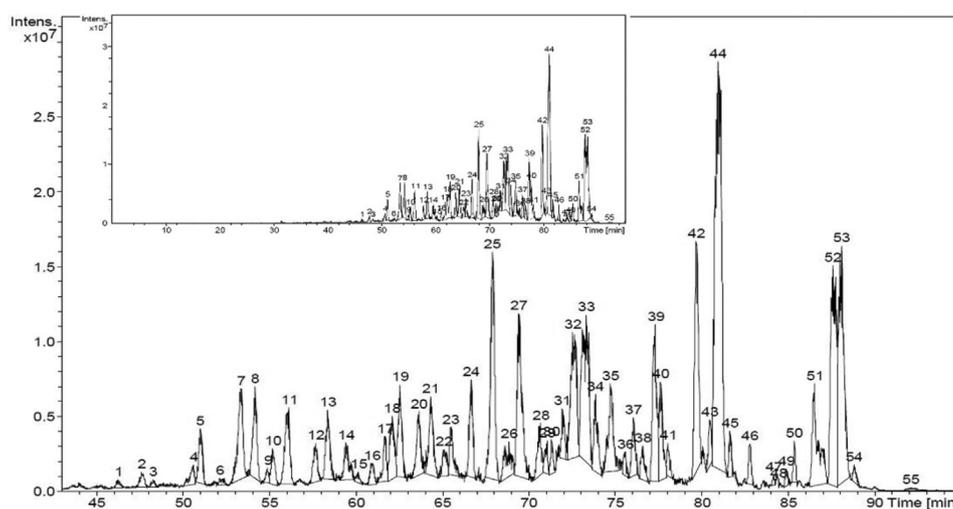


Fig. 1. HPLC-ESI-MS chromatogram of the Supermastic ES.

48 h, data not shown). Then cell medium was replaced with extract free medium and cell cultured for at least 1 week. Cells were then fixed and stained with crystal violet. Only colonies > 50 cells were counted. Each experiment was performed in triplicate and repeated 2 times.

2.6. Cell morphology analysis by Wright staining

Caco-2 cells (5×10^4 cells/well in 24-well plates) were grown on coverslips for 48 h and then treated with ES 50, 100 $\mu\text{g}/\text{ml}$ and 500 $\mu\text{g}/\text{ml}$ for 6 h (and 24 h, data not shown). Treated cells were fixed in methanol for 5 min, stained with Wright stain and observed under a light microscope for evaluation of cell morphology as previously described (Rubin et al., 2018). Experiments were performed in duplicate and repeated 2 times.

2.7. Cell cycle analysis

Caco-2 cells were plated into 25 cm² flasks at a density of 1×10^6 cells/well and were treated with ES at 50 or 500 $\mu\text{g}/\text{ml}$ for 6 h, then trypsinized and harvested by centrifugation. Cells were resuspended in ice-cold PBS and fixed in 70% ice-cold ethanol, followed by an overnight incubation at -20°C . After washing, cells were stained with propidium iodide solution (50 $\mu\text{g}/\text{mL}$ of propidium iodide plus 10 $\mu\text{g}/\text{mL}$ RNaseA) and incubated for 1 h at 37°C in the dark. Data were acquired by CytoFLEX (Beckman Coulter, United States) and analysed by CytExpert software (Beckman Coulter, United States). Results were analysed by *t*-test comparing the percentage of events in each phase through treatments. Experiments were performed in triplicates.

2.8. Interleukin-6 (IL-6), Interleukin-1 α (IL-1 α), tumor necrosis factor (TNF α) quantification analysis

Caco-2 cells (1.0×10^6 cells/well in 6-well plates) were grown for 48 h and then treated with ES 50 or 500 $\mu\text{g}/\text{ml}$ for 6 h. The concentration of interleukins in cell culture supernatants was determined using a commercial ELISA kit for quantification of human interleukins IL-6, IL-1 α and TNF α (ELISA MAX Deluxe Set, BioLegend, Germany), according to the manufacturer's protocol. Each analysis was performed in triplicate and repeated 3 times.

2.9. Disaccharidase activity

Disaccharidase activity was indirectly measured in Caco-2 cells following a published method (Turco et al., 2016). Cells (2.5×10^5 cells/well in 6-well plates) were grown for 72 h and then treated or untreated with ES 50 or 500 $\mu\text{g}/\text{ml}$ for 6 h. The collected cells underwent ultrasonic disruption in glucose-free Hanks' balanced salt solution. A pre-warmed solution of maltose (56 mM) and sucrose (56 mM) in PBS was added to the cells and the well plates were incubated at 37°C for 60 min. The reaction was stopped by adding 0.1 ml of methanol and glucose generated by the enzymatic activity was measured according to the manufacturer's instructions (Glucose Colorimetric Detection Kit, Thermo Fisher Scientific, Milano, Italy). Each analysis was performed in triplicate and repeated 2 times.

2.10. RNA extraction, quantification, reverse transcription and quantitative Real-time PCR

Caco-2 cells (2.5×10^5 cells/well in 6-well plates) were grown for 72 h and then treated or untreated with ES 50 or 500 $\mu\text{g}/\text{ml}$ for 6 h. Then total RNA was extracted using the Direct-zol RNA Miniprep system (Zymo Research, CA, USA), according to the manufacturer's protocol. The concentration and integrity of the RNA and cDNA synthesis were checked. A real-time quantitative PCR (qRT-PCR) was performed in an ABI-PRISM 7900HT Sequence Detector (Applied Biosystems, Milano, Italy) as already described (Mariniello et al., 2012).

β -actin (housekeeping gene) (*ACTB* gene), IL-1 α (*IL1A* gene), IL-6 (*IL6* gene) and sucrase-isomaltase (*SI* gene) primer sequences were used as described elsewhere (Alkasalim et al., 2017; Cheng et al., 2014; Hossen et al., 2017). The β -actin primers (forward, 5'-GGGACGACATGGAGA AAATCTG-3', reverse, 5'-CACGCAGCTCATTGTAGAAGGT-3'), IL-1 α primers (forward, 5'-ACTGCCCAAGATGAAGACCA-3', reverse, 5'-CCG TGAGTTTCCAGAA-3'), IL-6 primers (forward, 5'- AATTCGGTAC ATCCTCGACGG-3', reverse, 5'- GGTTGTTTTCTGCCAGTGCC-3'), and sucrase-isomaltase primers (forward, 5'-CATCCTACCATGTCAAGAGC CAG-3', and reverse, 5'-GCTTGTTAAGGTGGTCTGGTTTAAATT-3') were purchased by the Invitrogen Life Technologies Company (Monza MB, Italy). Sucrase-isomaltase gene is specifically expressed by enterocytes and Caco-2 cells (Cheng et al., 2014). Data were analysed with the Sequence Detection Software rel. 2.4 (Applied Biosystems). Data were obtained as C_t values and used to determine ΔC_t values ($\Delta C_t = C_t$ of the target genes minus C_t of the housekeeping genes). The equation $2^{-\Delta\Delta C_t}$ was used to calculate the fold changes in gene expression as previously described (Mariniello et al., 2012). The experiment was performed in triplicate.

2.11. Paracellular permeability assay (PPA)

Caco-2 cell monolayers were grown on transwell permeable supports (pore size 0.4 μm ; Corning Costar) in 24-well plates for 4 weeks. After treatment of cell monolayers with ES 50 or 500 $\mu\text{g}/\text{ml}$ for 6 h, paracellular permeability was measured based on fluorescein flux across Caco-2 monolayers as previously reported (Balimane & Chong, 2005; Konishi, Hagiwara, & Shimizu, 2002). The basolateral chamber of the inserts was filled with 0.6 ml of Hank's balanced salt solution (HBSS; pH 7.4, 37°C), whereas 0.1 ml of 0.04% fluorescein was added on the apical side of the supports. Cell monolayers were incubated at 37°C and samples were collected from the basolateral side every 15 min, up to 90 min. The amount of fluorescein permeated through cells was estimated using a VICTOR X3 Plate Reader (PerkinElmer, Milano, Italy) at 485 nm excitation and 530 nm emission. The apparent permeability coefficient was finally calculated according to the following equation: $P_{app} = [AF]/t \times 1/A \times V/[LC]$, where [AF] is the abluminal concentration of fluorescein, *t* is time in seconds for fluorescein incubation, A is area of membrane in cm², V is the volume of the and [LC] is the luminal concentration of fluorescein. Each experiment was performed in triplicate and repeated 2 times.

2.12. Wound healing assay

Caco-2 cells were seeded on 6-well plates at 2×10^6 cells/well and treated for 6 h (and 24 h data not shown) with ES at 50, 100 and 500 $\mu\text{g}/\text{ml}$. At the end of the treatment, the medium was removed and a scratch wound was created using a pipette tip. Cell migration was analysed for the following 3 days. The variation in recovery of the wound area by migrating cells was recorded under an inverted microscope (40X) and digitally analysed at the end of the experiment. The experiments were run in quadruplicate.

2.13. Statistical analysis

All statistical analysis was performed using Prism software version 6 (GraphPad Software, Inc.). One-way ANOVA was used for data involving 3 or more groups with one variable followed by the Student's *t*-test to determine significant difference between treated and untreated samples. *P* values < 0.05 were considered statistically significant.

3. Results and discussion

This work analysed the effects of a standardized *Pistacia lentiscus* extract (ES) (Fig. 1) in a human small intestine mucosa cell model, Caco-2. First we studied the influence of ES in a cell viability assay

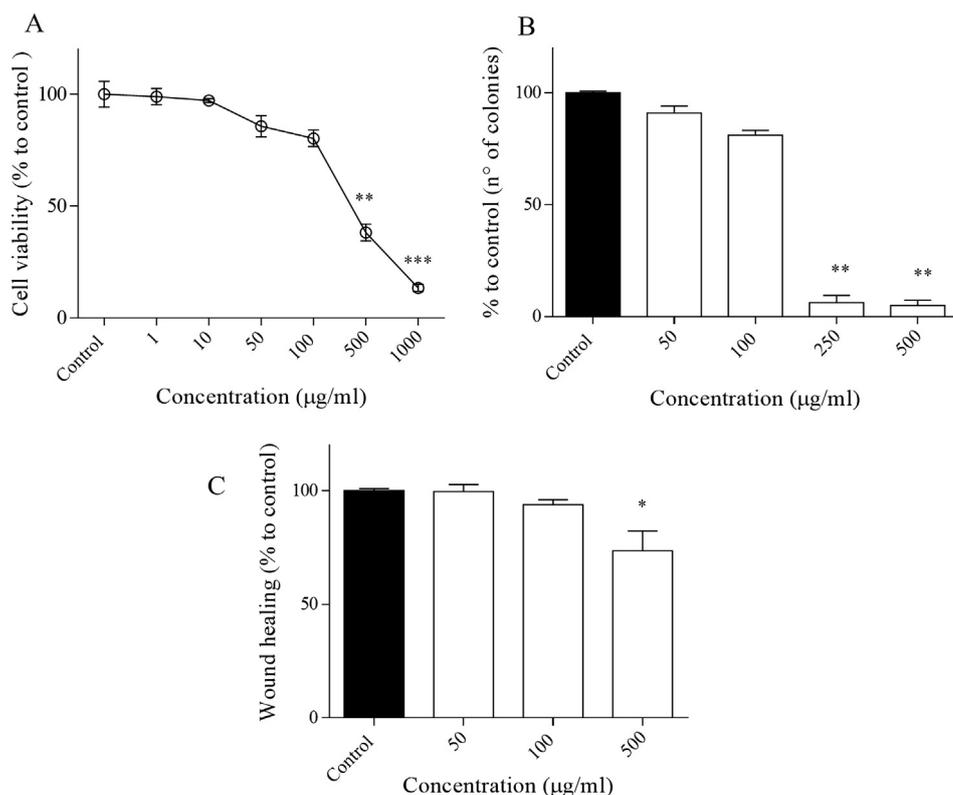


Fig. 2. Caco-2 cells treated with ES at different concentrations. (A) MTT test at 6 h. Experiments performed in triplicate. (B) Clonogenic assay at 24 h. Experiments performed in quadruplicate. (C) Histogram of wound healing assay. Experiments performed in quadruplicates. Treatment vs control: *t*-test, **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

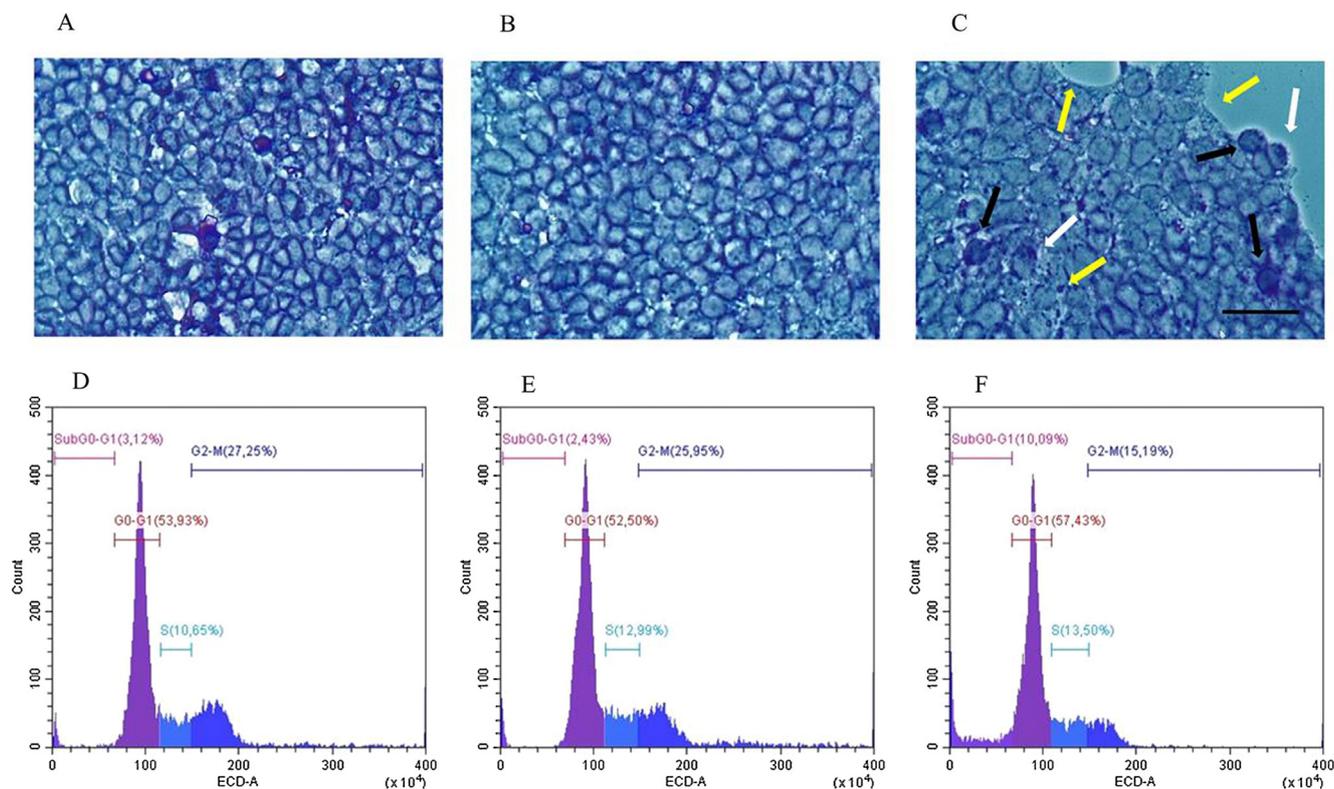


Fig. 3. Morphology and cell cycle analysis in Caco-2 cells. A-C) Cells morphology at 400X magnification with inverted microscopy. (A) Control untreated cells; (B) treated cells with ES at 50 µg/ml for 6 h; (C) treated cells with ES at 500 µg/ml for 6 h. Black arrows show apparently necrotic cells; white arrows show apparently apoptotic cells; yellow arrows show areas of apparent deterioration of the epithelium. Experiments performed in duplicate. D-F) Representative distribution of cell cycle analysis, (D) Control untreated cells. (E) treated cells with ES at 50 µg/ml for 6 h. (F) treated cells with ES at 500 µg/ml for 6 h. Experiments performed in triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(MTT test). We showed a decrease in cell viability at higher concentrations (> 100 µg/ml) and prolonged incubation time (> 6h), while at lower concentrations and reduced incubation time no significant modification was perceived (Fig. 2A and Fig. S1). By MTT test we determined the most potential effective concentration that did not induce cytotoxicity, possibly preserving ES biological effects (< 100 µg/ml). Similarly, clonogenic survival assay, that is based on the ability of a single cell to grow into a colony, showed that this ability was maintained at concentrations below 100 µg/ml, again remarking the non-toxic activity of ES at lower concentrations (Fig. 2B and Fig. S2). In addition, cell migration and cell–cell interaction were assessed by wound healing assay in Caco-2 cells. We showed that lower concentrations of ES (< 100 µg/ml) did not alter the ability to recover the gap and occupy the wound if compared to untreated cells (Fig. 2C and Fig. S3). Only ES at 500 µg/ml decreased this ability, with a probably harmful effect as reported in the viability experiments. Furthermore, morphological change in Caco-2 cells treated with ES was evaluated (Fig. 3A–C). It is known that numerous compounds or drugs can modify cell shape or cytoskeleton as a direct consequence on cell survival or growth (Domura, Sasaki, Ishikawa, & Okamoto, 2017; Futamura et al., 2012). We showed no appreciable alterations in cell morphology at 50 (Fig. 3B) and 100 µg/ml (not shown), with a constant confluent cell arrangement (cells occupy an ordered space), while at higher concentrations (500 µg/ml, Fig. 3C) treated cells demonstrated a lack of confluence (cells seem to assume an unordered position and leave empty spaces with presence of dead cells). Also cell cycle distribution was analysed in Caco-2 cells after ES treatments. If compared to control, ES 50 µg/ml did not significantly modify cell cycle phases (Fig. S4), while ES 500 µg/ml treatment caused an appreciable increase in subG0/G1 (related to cell death) and a concomitant decrease in G2/M phase (Fig. 3D–F).

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jff.2019.01.007>.

In order to study the inflammation process potentially induced by ES in Caco-2 cells, IL-1α, IL-6 and TNFα were quantified by a specific ELISA kit (Fig. 4A–C). It has been already reported that *Pistacia lentiscus* has anti-inflammatory activity (Dimas et al., 2012; Paraschos et al., 2012): correspondingly, no significant difference was perceived between ES 50 µg/ml and control in Caco-2 cells, suggesting that ES did not induce inflammation on this cell model. On the same line, mRNA levels of IL-1α and IL-6 quantified by Real-time PCR showed no increase of inflammatory cytokines (Fig. 4D and E). A complex picture emerges and only one assumption can be done: more work is needed to elucidate the apparent different role of cytokines in human and animals. Moreover, we showed an uncommon decrease of IL-1α and this result needs to be further investigated with more particular biomolecular tools (expression vector, specific inhibition, etc.).

In addition, we investigated whether ES could act on disaccharidases: the enzymatic activity of sucrase and isomaltase was therefore analysed in Caco-2 cells. Treatments with ES 50 and 500 µg/ml significantly decreased enzymatic activity, implying that suppressed disaccharidases could be strictly related to hypoglycaemic effect (Fig. 4F), as reported in different works (Georgiadis et al., 2014; Kartalis et al., 2016; Paraschos et al., 2012; Triantafyllou et al., 2007). Furthermore, modulation of disaccharidase activity induced by ES was explored and related to different gene expression. mRNA levels of sucrase-isomaltase was investigated by quantitative Real-time PCR and the results showed that also mRNA levels were significantly reduced suggesting that ES could deeply impact on gene expression, corroborating hypoglycaemic effect of ES observed by suppressed disaccharidase activity and literature data (Georgiadis et al., 2014) (Fig. 4G). We described for the first time a potential mechanism of action of ES, which is represented both at protein and genetic level.

Moreover to investigate whether ES could affect paracellular permeability, fluorescein flux across cell layers was measured with or without ES treatment in Caco-2 cell monolayer. The results showed a

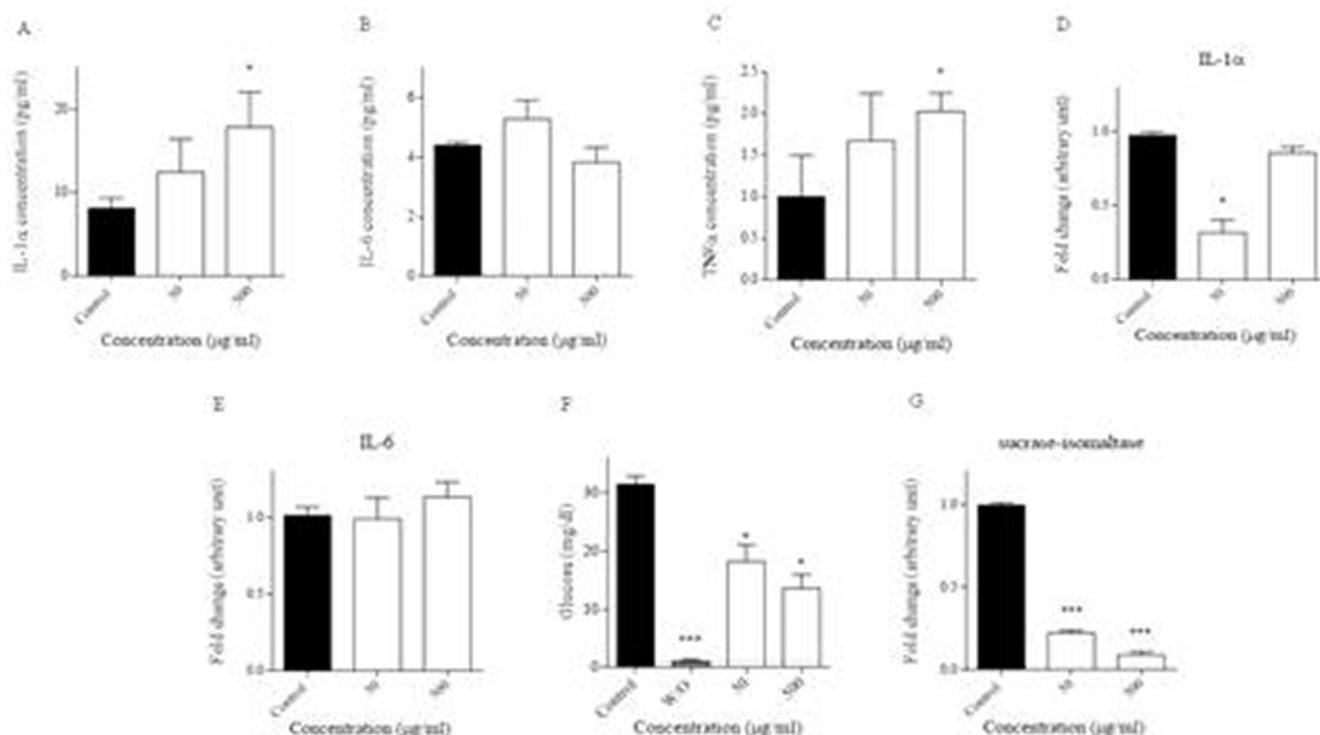


Fig. 4. Inflammation and disaccharidase in Caco-2 cells. (A–C) Interleukins quantification analysis in Caco-2 cells treated or untreated with different concentrations of ES. Experiments performed in triplicates. (D and E) Quantitative Real-time PCR for IL-1α and IL-6. Experiments performed in triplicates. (F) Disaccharidase activity expressed as glucose generation in cells treated or untreated with different concentrations of ES. W/O: no disaccharidase added. Experiments performed in duplicates. (G) Quantitative Real-time PCR for sucrase-isomaltase. Experiments performed in triplicates. Treatment vs control: t-test, * p < 0.05, *** p < 0.001.

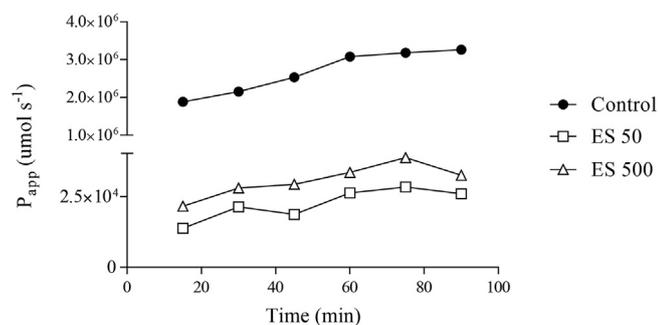


Fig. 5. Paracellular permeability assay in Caco-2 cells treated or untreated with different concentrations of ES. All results (treatments vs control) are significantly different, $p < 0.01$. Experiments performed in triplicates.

decrease in paracellular permeability of epithelial cells, in line with the above experiments which suggested a protective role for ES (Fig. 5). This role has been observed in different clinical trials involving patients affected by digestive system diseases (Dabos et al., 2010; Dabos et al., 2010; Kaliora et al., 2007; Kartalis et al., 2016; Triantafyllou et al., 2007): we hypothesize that ES could produce a sort of semi-permeable barrier above the epithelial layer, whose function needs to be demonstrated at molecular level. More work is needed to address this assumption.

In conclusion *Pistacia lentiscus* Chios mastic extract showed no toxic effects in Caco-2 cells treated with lower concentrations ($< 100 \mu\text{g/ml}$) as underlined by results of MTT test, clonogenic assay, morphology analysis, cell cycle distribution and wound healing assay. In addition, this extract did not trigger an increase in inflammation process, as observed by no altered interleukins quantification if compared to control. Yet disaccharidases activity was suppressed as well as *SI* gene expression, denoting a role for ES in glycaemic control. Interestingly this extract influenced paracellular permeability of Caco-2 cell monolayer: physiological permeability of epithelial cells was decreased, suggesting a barrier effect useful in protecting against chemical or biological insults.

Conflict of interest

The authors declare no conflict of interest.

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Ethics statement

This research work did not include any human subjects and animal experiments.

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