Article

Unveiling the differential anti-oxidant activity of maslinic acid in murine melanoma cells and in rat embryonic healthy cells following treatment with hydrogen peroxide

Khalida Mokhtari[1,2], Amalia Pérez-Jiménez[3], Leticia García-Salguero[1], José A. Lupiáñez[1] and Eva E. Rufino-Palomares [1*]

[1] Department of Biochemistry and Molecular Biology I, Faculty of Sciences, University of Granada, Avenida Fuentenueva, 1, 18071 Granada, Spain; khalidafadoua@hotmail.com (K.M.); elgarcia@ugr.es (L.G.-S.); jlcara@ugr.es (J.A.L.).

[2] Department of Biology, Laboratory of Bioresources, Biotechnologies, Ethnopharmacology and Health, Faculty of Sciences, Mohammed I University of Oujda, 60000 Oujda, Morocco

[3] Department of Zoology, Faculty of Sciences, University of Granada, Avenida Fuentenueva, 1, 18071 Granada, Spain; calaya@ugr.es (A.P.-J.).

* Correspondence: evaevae@ugr.es (E.E.R.-P.); Tel.: +34 958 243252.

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Abstract: Maslinic acid (MA) is a natural triterpene from *Olea europaea* whose pharmacological functions have been showed. The objective of this study was to examine MA effect on cell viability (by MTT assay), reactive oxygen species (ROS levels, by flow cytometry) and key anti-oxidant enzyme activities (by spectrophotometry) in murine skin melanoma (B16F10) cells compared to healthy cells (A10). MA induced cytotoxic effects in cancer cells (IC_{50} 42 μM) whereas no effect was found in A10 cells treated with MA (up to 210 μM). In order to produce a stress situation in cells, 0.15 mM of H_2O_2 were added. Under stressful conditions, MA protected both cell lines against oxidative damage, decreasing intracellular ROS, being higher in B16F10 than in A10 cells. The treatment with H_2O_2 and without MA produced different responses in anti-oxidant enzymes activities depending on cell line. In A10 cells, all enzymes were up-regulated, but in B16F10 cells only superoxide dismutase, glutathione S-transferase and glutathione peroxidase increased their activities. MA restored the enzyme activities to similar levels than control group in both cell lines, highlighting that in A10 cells the highest MA doses induced values lower than control. Overall, these findings demonstrate the great anti-oxidant capacity of MA.

Keywords: anti-oxidant activity; anti-oxidant enzymes, anti-proliferative activity; maslinic acid; melanoma; *Olea europaea*; ROS levels.

1. Introduction

Oxidative stress is defined as an imbalance between pro-oxidant and anti-oxidant molecules that modify the normal cell physiology due to proteins, lipids, carbohydrates and nucleic acids damage [1]. Many cellular processes depend on the variations in the levels of ROS and NADPH that take place during their development and that, fundamentally, are determined by the activity of the different production systems of this reduced coenzyme, especially those belonging to the pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) and, also, to NADP-dependent isocitrate dehydrogenase (ICDH-NADP). The cellular redox state will be key to interpreting the behaviour of most of these key cellular

processes for the vital development of organisms, such as cell differentiation [2-4], cellular growth [5-8], nutrition cell [6, 9-11] and cell aging [12].

Normal cellular metabolism is well established as the source of endogenous pro-oxidants and the most important are reactive oxygen species (ROS) and reactive nitrogen species (RNS) [13]. Under these normal circumstances, ROS are neutralized by an anti-oxidant defense system consisting of enzymes such as, catalase (CAT), which reduces hydrogen peroxide; superoxide dismutase (SOD), which detoxifies superoxide radical; glutathione peroxidase (GPX), which reduces hydrogen peroxide and other organic peroxides; S-transferase glutathione (GST), which detoxifies harmful molecules; glutathione reductase (GR), which regenerate glutathione (GSH) from its oxidized form (GSSG) by NADPH-dependent pathway; G6PDH which produces NADPH for GR mechanism and also is a direct scavenger of free radicals [14, 15] and numerous non enzymatic anti-oxidant molecules [16]. However, although a certain level of DNA damage cannot be avoided, oxidative stress can occur when the balance is upset, either by excessive production of ROS, by deficient anti-oxidant defences, or by a combination of both [17]. In cancer cells, oxidative stress has been linked to the regulation of numerous cellular processes, including DNA damage, proliferation, cell adhesion, migration, and regulation of cell survival or cell death [15].

Hydrogen peroxide (H_2O_2) is originated from $O_2\bullet$- by SOD. It is not a free radical as such, but it is a reactive form, since it has the capacity to generate the hydroxyl radical in the presence of metals such as iron [1]. H_2O_2 is a key metabolite in oxidative stress being an important representative ROS that arises during the aerobic respiration process and from other cellular sources [18]. As a messenger molecule, H_2O_2 diffuses through cells and tissues to initiate immediate cellular effects, such as cell shape changes, initiation of proliferation and recruitment of immune cells. If not controlled, an excess of H_2O_2 can provoke a condition of decontrolled oxidative stress in the cells producing irreparable damage [19]. Therefore, cell death and survival processes can be modulated controlling intracellular H_2O_2 levels by using anti-oxidants. The appeal of natural products has stemmed from the cost-effectiveness, fewer side effects of natural products and for being an inexhaustible source. As such, products like maslinic acid (MA) have received heightened interest.

MA, $C_{30}H_{48}O_4$ (2α, 3β-dihydroxyolean-12-en-28-oic acid) is natural pentacyclic triterpene, also known as crataegolic acid and derived from its structural analogue, oleanolic acid (Figure 1). It is a compound formed by 30 carbon atoms grouped into five cycles that, as substituents, have two methyl groups on the C-4 and C-21 carbons; a methyl groups at the C-8, C-10 and C-15 carbons; two hydroxyl groups on the C-2 and C-3 carbons; a carboxyl group at the C-28 carbon and a double bond on the C-12 and C-13 [20-23].

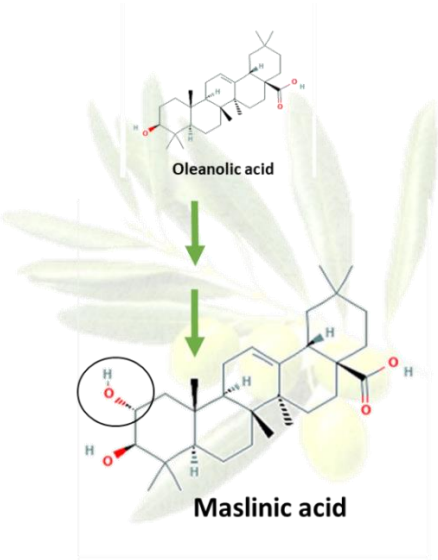

Figure 1. Schedule chemical structures of oleanolic acid and maslinic acids from pomace olive (*Olea europaea* L.) (Pubchem).

Maslinic acid has been isolated from various plants, being especially abundant in *Crataegus oxyacantha* and in the surface wax of fruits and leaves of *Olea europaea* and the solid waste from olive oil extraction [24]. Furthermore, MA is among the main triterpenes present in olives and olive oil. Its concentration in the oil depends on the type of olive oil and the variety of olive tree [23].

MA has a high pharmacological interest, highlighting its anti-tumour effect in certain types of cancer besides its anti-inflammatory, anti-oxidant and anti-diabetogenic, anti-hypertensive, anti-viral and cardioprotective effects, among others [1, 23]. Among the bioactivities attributed to MA, its anti-oxidant effect is the most contradictory. Montilla et al. [25] observed that MA reduced the plasma levels of endogenous lipid peroxides and the susceptibility of plasma to lipid peroxidation in a model of oxidative status induced by CCl_4. Similarly, MA isolated from the flowers of *Punica granatum* prevented the $CuSO^{4-}$ induced oxidation of rabbit plasma LDL [26]. Allouche et al. [27] performed a study about the anti-oxidant properties of several pentacyclic triterpenic diols and acids on LDL particles isolated from human plasma. Particularly, MA not only retarded the initiation and decreased the rate of $CuSO^{4-}$ induced LDL oxidation, but also showed peroxyl radical scavenging activity and a slight metal (copper) chelating effect [27].

Further research has been done in macrophages, which play a role in the defensive system of the organism in response to activation by a pathogen [28]. Results of this study indicated that MA did not exert any direct inhibitory effects on the formation of NO and superoxide, but did reduce the generation of H_2O_2 (IC_{50} of 46.3 μM), in a way that was similar to that of catalase [29]. In a previous study, we concluded that MA produced an increase in the ROS level under stress conditions caused by absence of FBS in melanoma cell [15]. Furthermore, in this same study, MA had an anti-oxidant effect at lower assayed levels; however, at higher dosages MA induced cellular damage by apoptosis.

The objective of this study was to examine the anti-oxidant effect of MA in murine skin melanoma (B16F10) cells and in a healthy cell line derived from the thoracic aorta of embryonic rat (A10), by analyzing the proliferation, ROS production and the activity of the main anti-oxidants enzymes under cellular stress conditions induced by H_2O_2.

2. Results

2.1. MA inhibits proliferation of B16F10 cells by a dose-dependent mechanism

We examined the effect of MA on the proliferation of B16F10 melanoma and A10 cell lines using MTT assay (Figure 2 A and B). B16F10 and A10 cells were exposed to 0 to 212 μM of MA for 24 h and cell survival was then compared with untreated controls, as determined by formazan dye uptake. The percentage of living cells (viable formazan-accumulating cells) decreased concomitantly with dose in cancer cells (B16F10) while in healthy cells (A10), MA did not produce any cytotoxic effect, even at the highest doses used (210 μM). In B16F10 cells, we noticed that 50% growth inhibition values (IC_{50}) in response to MA were of 42 μM after 24 h of addition of this compound. Based on this value, the rest of studies were performed with an exposition of cells to 10.6 μM ($IC_{50/4}$), 21.2 μM ($IC_{50/2}$), 42.3 μM (IC_{50}) and 84.6 μM ($2{\cdot}IC_{50}$) of MA for 24 h.

2.2. H_2O_2 modifies cell viability

We examined the effect of H_2O_2 on cell viability of B16F10 and A10 cells in order to know the optimal dose capable of producing a stress without reaching the cell death (Figure 2 C and D). Cells were exposed to 0 to 3 mM of H_2O_2 for 24 h and cell survival was then compared with untreated controls, as determined by formazan dye uptake. The percentage of living cells decreased concomitantly with dose in both cell lines until the concentration of 1.5 mM from which H_2O_2 had no cell viability effect. In both cell lines, we noticed an IC_{50} of 0.2 mM. In no case, with the doses used and the incubation time employed, any mortality higher than 80% was reached. Based on these results, the concentration of 0.15 mM of H_2O_2 was used to perform the rest of studies.

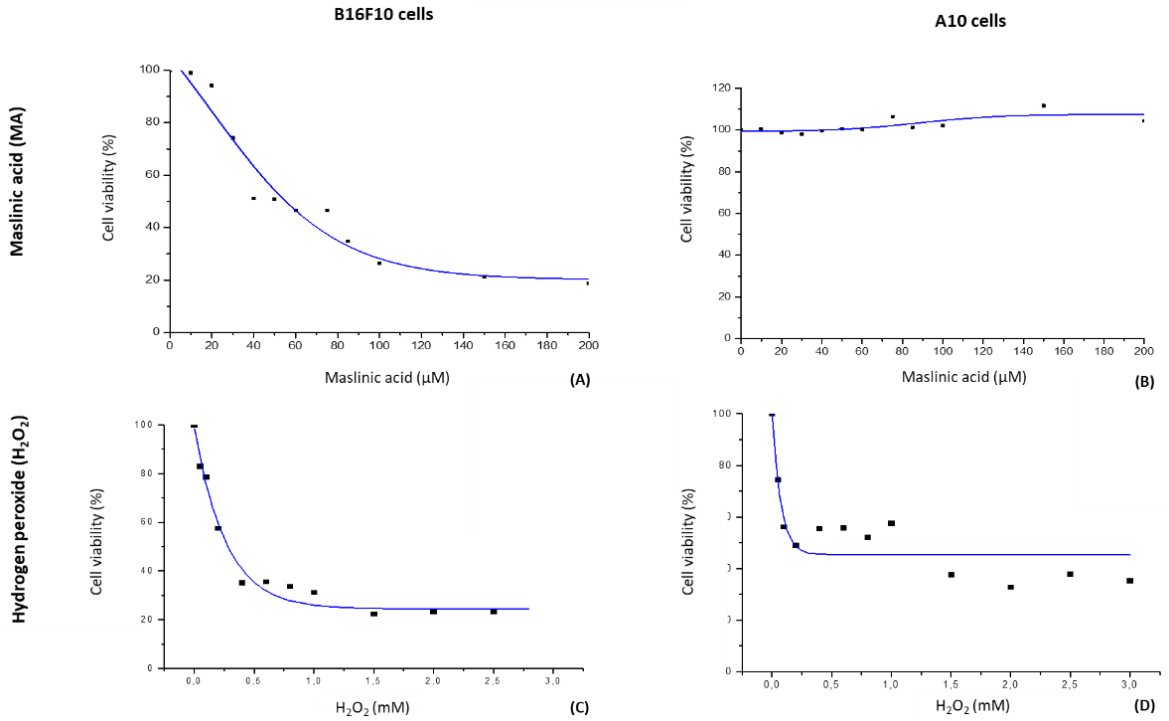

Figure 2. Cytotoxicity curves of maslinic acid (MA) on B16F10 murine melanoma cells (A) and A10 rat embryonic healthy cells (B) and hydrogen peroxide (H_2O_2) on B16F10 murine melanoma cells (C) and A10 rat embryonic healthy cells (D). Cell proliferation was determined by MTT assay. Values are expressed as means ± SEM (n = 9).

2.3. Maslinic acid influences on mitochondrial-membrane potential

We performed an assay of ROS production by DHR. Firstly, we tested ROS levels that occur over time in the presence of 0.150 mM of H_2O_2 and using different MA levels ($IC_{50/4}$, $IC_{50/2}$, IC_{50} and $2{\cdot}IC_{50}$) based on our cytotoxicity results. The results are showed in Figure 3. After 24 h of H_2O_2 treatment, ROS levels increased significantly in both cell lines, compared to the controls. The increment observed by H_2O_2 addition was offset by MA supplement, which produced a decreased in the intracellular ROS levels at any MA levels used ($IC_{50/4}$, $IC_{50/2}$, IC_{50} and $2{\cdot}IC_{50}$) in B16F10 and A10 cells. Moreover, in A10, ROS levels decreased below the value of control cells for all concentrations of MA tested.

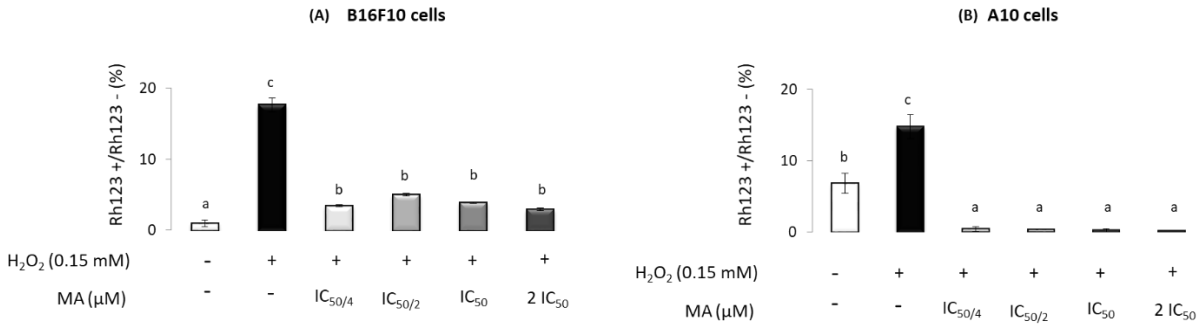

Figure 3. Positive fluorescent Rh123 on B16F10 cells (A) and A10 cells (B) with (+) or without (-) H_2O_2 and MA treatment at different dosages, $IC_{50/4}$, $IC_{50/2}$, IC_{50}, and $2{\bullet}IC_{50}$ (10.6, 21.6; 42.3 and 84.6 μM, respectively. Values are expressed as mean ± SEM (n = 9). Different letters indicate significant differences ($p < 0.05$).

2.4. MA exerts as anti-oxidant activity modulating enzymatic defense system

An evaluation of the activities of the anti-oxidant enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST), catalase (CAT), glucose 6-phosphate dehydrogenase (G6PDH), glutathione peroxidase (GPX) and glutathione reductase (GR) was performed under our experimental conditions.

Results obtained for SOD in B16F10 cells showed that H_2O_2 increased its activity and MA (at any level) decreased, but without significant differences induced by different MA concentrations. In A10, H_2O_2 increased the SOD activity, increment that was mitigated with MA addition. Moreover, 42.3 and 84.6 µM of MA (IC_{50} and 2·IC_{50}, respectively) resulted on SOD activity levels lower than those found in absence of H_2O_2 and MA (Figure 4 A and B).

In B16F10 cells, GST activity increased in presence of H_2O_2, whereas the incubation with $IC_{50/4}$ and $IC_{50/2}$ of MA decreased the levels making them equal to the control without H_2O_2 and MA. The use of IC_{50} and 2·IC_{50} MA concentrations induced GST activities lower than control. Similar results were found in A10 for GST activity, with the exception of MA at $IC_{50/2}$ that also induced lower levels than control (Figure 4 c and d).

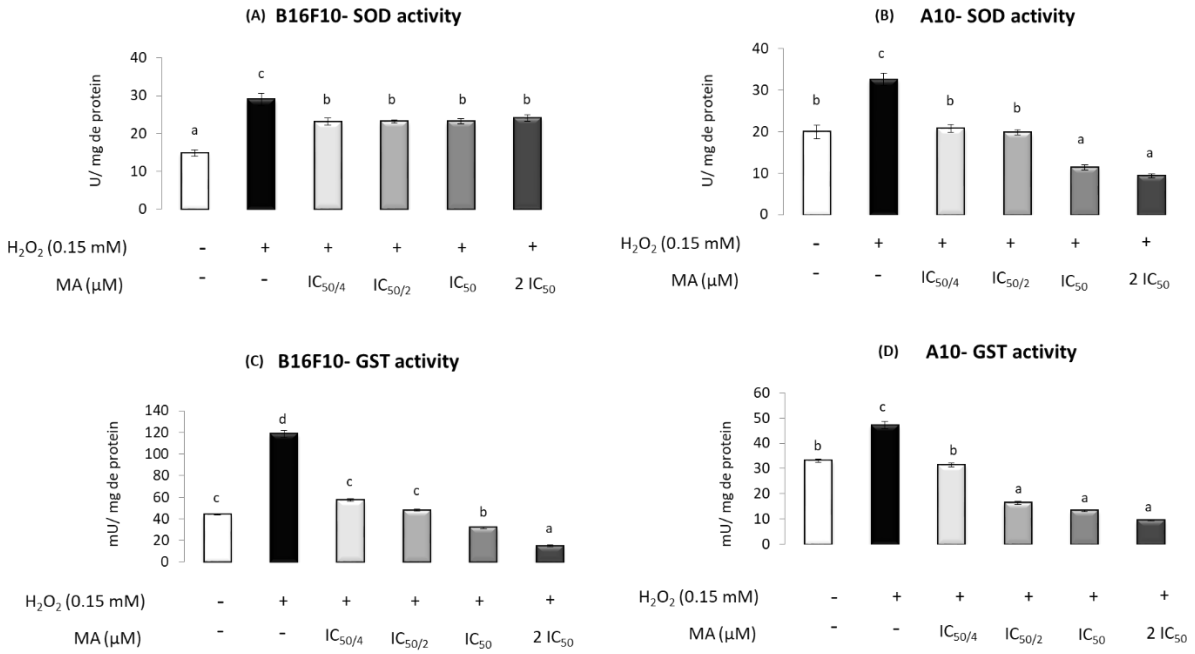

Figure 4. Effect of maslinic acid on the specific activity of Superoxide dismutase (SOD) in cancer cells, B16F10 5(A), and normal cells, A10 (B), and glutathione S-transferase (GST) in cancer cells, B16F10 (C), and normal cells, A10 (D), subjected to the presence of hydrogen peroxide. Enzyme activities (U or mU × mg protein^{-1}) are expressed as mean ± SEM (n = 9). Different letters indicate significant differences ($P < 0.05$).

In B16F10 cells, CAT activity decreased in presence of H_2O_2 compared to the control, whereas MA increased the activity reaching values higher or similar than control when $IC_{50/4}$ or $IC_{50/2}$ were used. In A10 cells, H_2O_2 produced an increased in the CAT activity and its activity was not recovered until the highest doses of MA were used (IC_{50} and 2·IC_{50}) (Figure 5 A and B).

Regarding G6PDH, the results indicated its activity decreased in presence of H_2O_2 in B16F10 and MA in all concentrations tested had similar values to the control without H_2O_2 and MA. In A10 cells, no changes were induced by H_2O_2 in G6PDH, whereas MA decreased its activity at any tested level (Figure 5 C and D).

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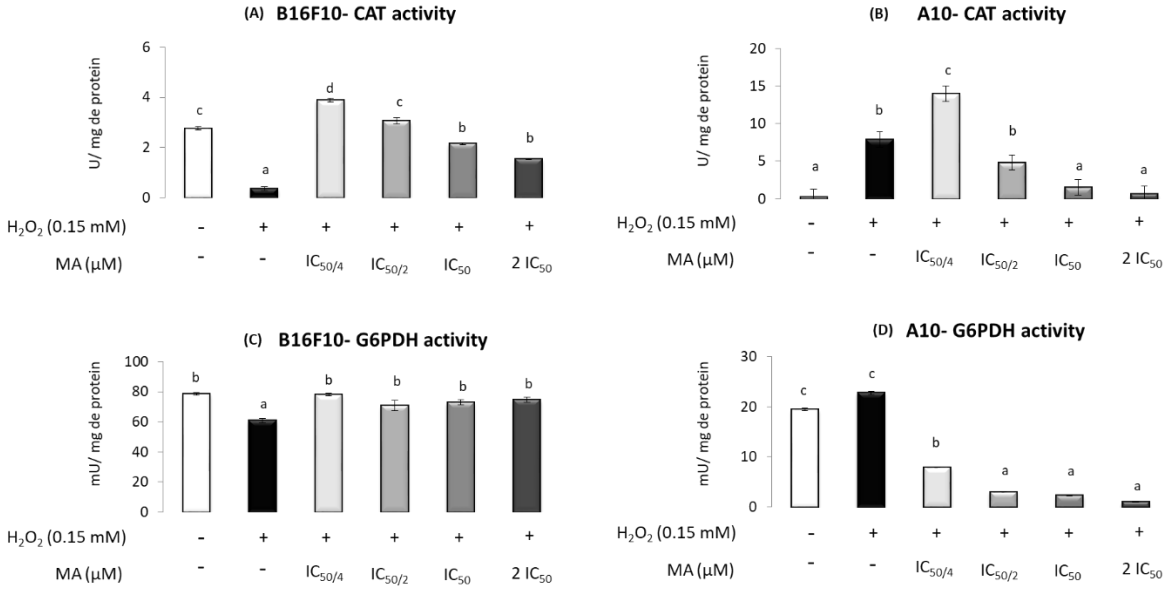

Figure 5. Effect of maslinic acid on the specific activity of catalase (CAT) in cancer cells, B16F10 (A), and normal cells, A10 (B), and glucose 6-phosphate dehydrogenase (G6PDH) in cancer cells, B16F10 (C), and normal cells, A10 (D), subjected to the presence of hydrogen peroxide. Enzyme activities (U or mU × mg protein^{-1}) are expressed as mean ± SEM (n = 9). Different letters indicate significant differences (P <0.05).

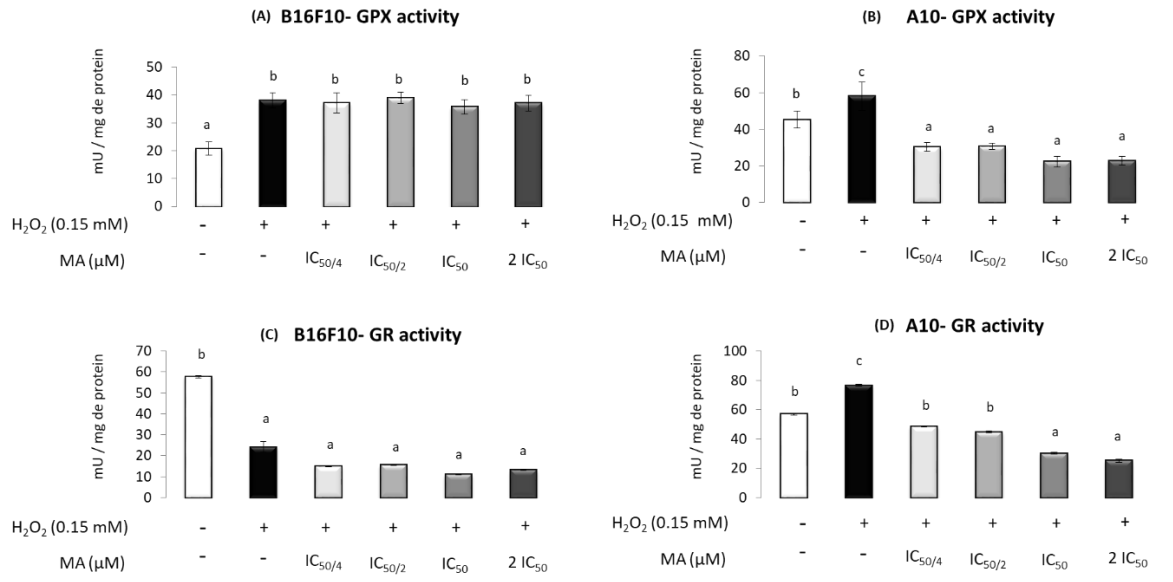

Figure 6. Effect of maslinic acid on the specific activity of glutathione peroxidase (GPX) in cancer cells, B16F10 (A), and normal cells, A10 (B), and glutathione reductase (GR) in cancer cells, B16F10 (C), and normal cells, A10 (D), subjected to the presence of hydrogen peroxide. Enzyme activity (mU × mg protein^{-1}) are expressed as mean ± SEM (n = 9). Different letters indicate significant differences (P <0.05).

GPX increased in presence of H_2O_2 but any change was observed when MA was administrated in B16F10. In A10 cells, all concentrations of MA decreased GPX activity increased by the H_2O_2 even below the control without H_2O_2 and MA (Figure 6 A and B).

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In B16F10, GR activity was decreased by H_2O_2 and no changes were observed at any MA level, maintaining lower levels of activity compared to the control. In A10, H_2O_2 increased GR activity, increment that was mitigated with MA addition. Moreover, 42.3 and 84.6 μM of MA induced values of GR activity lower than those found in absence of H_2O_2 and MA (Figure 6 C and D).

3. Discussion

Olive oil from *Olea europaea* is a common component in the diet of the Mediterranean countries. It has been showed that this natural source of nutrients presents very important beneficial properties for health due to their chemical composition. The pentacyclic triterpenes are especially abundant in the surface wax of fruits and leaves of tree. In this sense, maslinic acid (MA) in the main triterpene included in this family of molecules with demonstrated properties that include anti-cancer, anti-inflammatory, anti-viral, anti-allergic, anti-diabetogenic effects, among others [23, 30, 31]. Due to the large amount of bioactivities, these molecules are appreciated as chemopreventive agents in pathophysiological diseases such as cancer [23, 27], cardiovascular and neurodegenerative diseases, such as atherosclerosis, also as well in biological process like aging [25].

The effect cytotoxic of MA has been studied in different cell lines, including both cancer and healthy cells. In this sense, it has been showed that this compound has a variable effect depending on the type of cells and experimental conditions. In the present study, MA showed cytotoxic effects only in B16F10 cells and this effect was important, as the IC_{50} of MA was 42 μM. In another study performed in melanoma cells by Kim et al. [32], the authors observed a lower MA cytoxicity effect. So, they concluded that IC_{50} for MA in SK-MEL-3 was also 42 μM, but in their study the incubation period was 48h *versus* the 24h used in the present study. Other results obtained in our research group showed that in colon cancer cells, the IC_{50} value for MA was 30 μM in HT29 cells after 72h of incubation [31, 33, 34], showing a higher cytotoxic effect in Caco-2 cells in which the IC_{50} of MA was 10.82 μM, also after 72 h [35]. Other authors observed IC_{50} values for MA ranging between 32 μM and 64 μM in different cancer cells such as lung (A549), ovary (SK-OV-3), colon (HCT15) and glioma (XF498) after 48h of incubation [32]. In several bladder cancer cell lines incubated with MA during 48h, other authors found IC_{50} values between 20 and 300 μM [36]. Notwithstanding, compared to melanoma cells, non-cytotoxic effect was found in A10 healthy cells in the present study. These results are relevant since demonstrate the selective cytotoxic effect of this compound. Studies focused on the selective effect of MA are scarce. Reyes et al. [22] observed that epithelial intestinal cells incubated with 30 μM of MA during 72h (IC_{50} value for HT29) exhibited a survival rate of 78% in IEC-6 cells and 68% in IEC-18.

An intracellular redox balance is crucial to ensure the viability, growth and diversity of cell functions [17]. An excess of ROS is related to pathological processes producing oxidative damage when the anti-oxidant defense system is not able to counteract it. Hence, there is a growing interest of the scientific community and industry to obtain substances of natural origin aimed at preventing these pathologies and alterations caused by ROS. In this context, it is important to characterize the protective biochemical functions of natural anti-oxidants and to study their intracellular pathway signalling. A large number of plant constituents, such as MA, have anti-oxidant properties [37].

The present study examined the anti-oxidant effects of MA in skin melanoma cancer cells (B16F10) and thoracic aorta of embryonic rat cells (A10). The major findings were that MA improves oxidative stress caused by a H_2O_2 excess decreasing the intracellular ROS levels in both cell lines. H_2O_2 was used in this study because it is known that when is presented in excess, is one of the major compounds that can damage the cells [38]. Other authors have showed clearly the influence of H_2O_2 on the viability and ROS production of different cell types that were subsequently treated with other anti-oxidant natural compounds that reversed the oxidative damage caused by H_2O_2 [39, 40]. Furthermore, similar studies than us with others ROS producing molecules (i.e. CCl_4) observed that MA counteracted the lipid peroxidation generated in the nucleus [25]. Similarly, MA prevented the $CuSO_4$ induced oxidation in rabbit plasma LDL [41]. Moreover, Yang et al. [42] evaluated the anti-

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oxidant effects of MA derivatives that showed radical-scavenging activity and inhibition in NO production in RAW 264.7 cells.

Regarding MA effect on anti-oxidant enzymes in B16F10 cell line, as expected, SOD, GST and GPX activities were increased in response to the H_2O_2 addition. However, H_2O_2 decreased CAT, G6PDH and GR activities. H_2O_2 excess is responsible of the cellular damages that include imbalance in membranes and biomolecule alterations. This fact supposes an energetic extra cost for the maintenance of cellular homeostasis in order to repair the damaged produced. Both, cellular damages and energy requirements could affect to G6PDH activity, either for the impaired glucose transporters or enhanced glycolysis pathway, decreasing the available glucose for the pentose phosphate pathway. The regulation of NADPH levels is essential to understand the behaviour of numerous physiological processes being especially important for growth and cell differentiation [2], but also for anti-oxidant processes, among others. NAPDH is mainly generated by G6PDH enzyme and is required for the GPX and CAT activity involved in H_2O_2 removal pathway. So, CAT is protected of its inactivation by NADPH. Moreover, this reduction equivalent is used by GR to regenerate the oxidized glutathione to its reduced form required for GPX activity [43, 44]. The lower G6PDH activity observed when H_2O_2 was added could result in a decrease in available NADPH levels which might justified the reduction of CAT and GR activities. Similar results have been observed in previous studies in B16F10 cells submitted to stress conditions induced by FBS absence [15]. Mokthari et al. [15] reported low activity levels in CAT that were produced by the low NADPH levels due to the imbalance in the cellular homeostasis.

It has been established that MA is a compound with anti-oxidant capacity [15, 25, 27].When MA was added to the B16F10 cells, the changes induced by H_2O_2 excess in SOD and GST activities were counteracted. In this sense, scavenger MA effect reduced ROS levels and subsequently the need for acting these anti-oxidant defences. Moreover, the observed decreased ROS levels due to MA would involve a lower cellular damage, which would reverse the possible mechanisms responsible for the decrease G6PDH when H_2O_2 is added, raising its activity until the control values. This recovered G6PDH activity would produce the required NADPH level to prevent and reverse the down-regulation of CAT [43]. Finally, a dose-response effect was observed both in GPX and CAT, highlighting the anti-oxidant behaviour of MA in these cells.

Results found in this work, in A10 healthy cells, revealed that the all enzyme activities increased in stressful conditions originated by the H_2O_2 addition, except in G6PDH whose activity was slightly higher. MA in these cells restored the anti-oxidant enzyme activities reaching values similar to the control group in cells treated with the lowest MA levels ($IC_{50/4}$). Furthermore, when cells were treated with the two highest doses of MA (IC_{50} and $2{\cdot}IC_{50}$), the activity of all anti-oxidant enzymes, with the exception of CAT, decreased below control levels. This fact, confirms the relevant anti-oxidant effect of MA [15, 27, 43].

A summary of the biological properties of the MA in relation to its cytotoxic and proliferative effect as well as its antioxidant activity in cancer and healthy cells can be seen in Figure 7.

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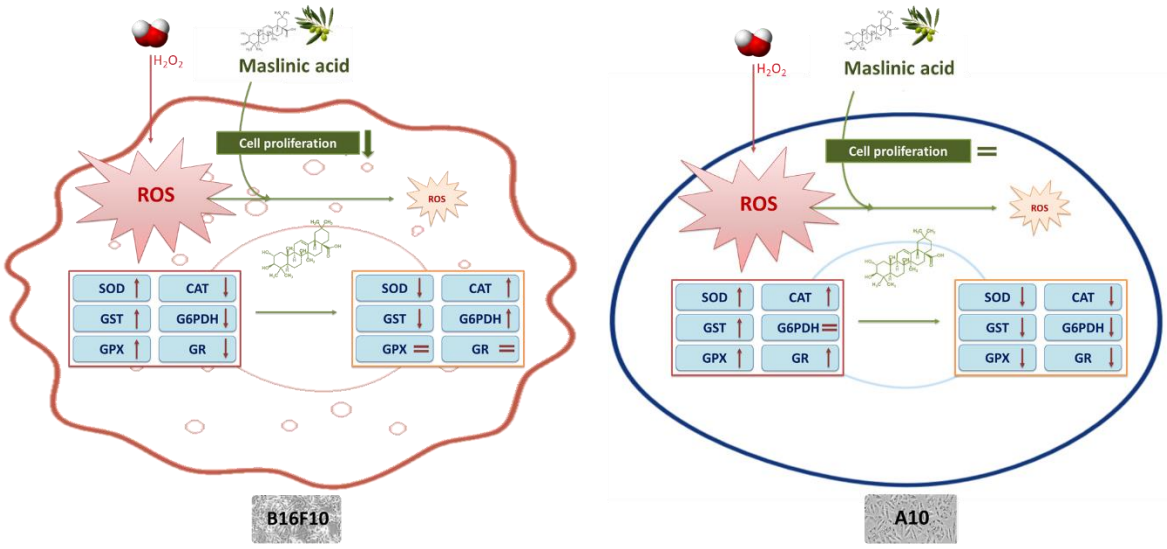

Figure 7. Scheme of the metabolic changes, in the proliferation processes and in the activity of the antioxidant system enzymes, that occur in cancer (B16F10) and healthy (A10) cells in response to treatment with H_2O_2 and MA.

4. Materials and Methods

4.1. Compounds

Maslinic acid was obtained from olive pomace and kindly donated by Biomaslinic S.A. Granada, Spain. The extract is a chemically pure white powder composed of 98% maslinic acid and stable when stored at 4 °C. It was dissolved before use at 10 mg/mL in 50% dimethyl sulfoxide (DMSO) and 50% phosphate buffer solution (PBS). This solution was diluted in cell culture medium for assay purposes.

4.2. Cell lines and cultures

Mouse melanoma cells, B16F10 is a variant of the murine melanoma cell B16. These cells show a higher metastatic potential than B16 cells [45]. A10 is a cell line derived from the thoracic aorta of embryonic mouse and it is used as a study model of smooth muscle cells (SMC). This cell type shows a great proliferative capacity and may be subcultured several times, allowing rapid attainment of cell mass) [46]. Both cell lines used were provided by the cell bank of the University of Granada (Spain). Cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing glucose (4.5 g/L) and L-glutamine (2 mM) from the commercial PAA home, 10% heat-inactivated foetal bovine serum (FBS) and 0.5% gentamicin to B16F10 cells and 10,000 units/mL penicillin, and 10 mg/mL streptomycin to A10 cells. The cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cells were passaged at preconfluent densities by the use of a solution containing 0.05% trypsin and 0.5 mM EDTA. Cells were seeded in the culture dishes at the desired density. After 24h hours, when the cells are attached to the dish, the cells were incubated with 0.15 mM of hydrogen peroxide (H_2O_2), in order to produce a stress situation into cells. Following, cells were incubated with several concentrations of MA as indicated below.

4.3. MTT assay

MTT assay was performed as described by Mokhtari et al. [15]. Briefly, 200 µL cell suspension of B16F10 or A10 ($1.5 \cdot 10^3$ cells/well) were cultured in 96 well plates. Subsequent to the adherence of the cells, different MA dilutions on a scale of 10 to 210 μM were added separately. Incubation times were 24 h for all cases. MTT was dissolved in the medium and added to the wells at a final concentration of 0.5 mg/mL. Following 2 h of incubation, the generated formazan was dissolved in DMSO. Absorbance was measured at 570 nm in a multiplate reader (Bio-tek®). Absorbance was proportional to the number of viable cells. The MA concentration leading to 50% inhibition of cell proliferation (IC_{50}) was determined. The results are expressed as percentage of live cells compared with the control considered as 100% cell viability. Cell viability in B16F10 and A10 cells were also studied in presence of H_2O_2 by MTT assay. H_2O_2 was dissolved in the culture medium of the cell lines. The concentrations used were made extemporaneously and protected by light before use to prevent degradation of the compound. After 24 hours of incubation the medium was removed, adding fresh medium with different concentrations of H_2O_2 per well: 0; 0.05; 0.1; 0.2; 0.4; 0.6; 0.8; 1.1; 1.5; 2.0; 2.6 and 3 mM to a final volume of 200 µL for 24h.

4.4. Flow-cytometry analysis of the mitochondrial-membrane potential

Changes in the mitochondrial-membrane potential can be examined by monitoring the cell fluorescence after double staining with rhodamine 123 (Rh123) and propidium iodide (PI). Rh123 is a membrane-permeable fluorescent cationic dye that is selectively taken up by mitochondria directly proportional to the MMP (mitochondrial-membrane permeabilization) [47]. B16F10 and A10 cells (4×10^5 cells/well) were seeded on 12-well plates with 2 mL of medium and treated with 0,15 mM for 24 h and MA at $IC_{50/4}$, $IC_{50/2}$, IC_{50}, and $2 \cdot IC_{50}$ (10.6, 21.6; 42.3 and 84.6 µM, respectively) concentrations during 24 h more. Following the treatment, the medium was removed and a fresh medium with DHR, at a final concentration of 5 µg/mL, was added. After 30 min of incubation, the medium was removed and the cells were washed and resuspended in PBS with 5 µg/mL of PI. The intensity of fluorescence from Rh123 and PI was determined using an ACS flow cytometer (Coulter Corporation, Hialeah, FL, USA), at the excitation and emission wavelengths of 500 nm and 536 nm, respectively. The experiments were performed three times with two replicates per assay.

4.5. Anti-oxidant enzymes assays

In order to prepare the samples for analytic procedures, cells were homogenized in RIPA buffer. Immediately, cells were sonicated on ice for 5 min and maintained by moderate shaking at 4°C for 1 h. Every 15 min, the samples were moderately shaken in a vortex. The lysates were spun in a centrifuge at 10,000 g at 4°C for 15 min. All enzyme assays were carried out at 37°C using a Power Wave X microplate scanning spectrophotometer (Bio-Tek Instruments, USA) and run in duplicate in 96-well microplates. The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The enzymatic reactions were initiated by addition of the cell extract, except for SOD where xanthine oxidase was used. The millimolar extinction coefficients used for H_2O_2, NADH/NADPH, and DTNB [5,5-dithiobis (2-nitrobenzoic acid)] were 0.039, 6.22, and 13.6, respectively. The assay conditions were as follows:

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the ferricytochrome c method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c, and 0.024 IU/mL xanthine oxidase. Activity is reported in units of SOD per milligram of protein. One unit of activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the ferricytochrome c reduction rate [48].

Catalase (CAT; EC 1.11.1.6) activity was determined by measuring the decrease of hydrogen peroxide concentration at 240 nm according to Aebi [49]. The reaction mixture contained 50 mm potassium phosphate buffer (pH 7.0) and 10.6 mM of freshly prepared H_2O_2.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was determined at pH 7.6 in a medium containing 50 mM Hepes buffer, 2 mM $MgCl_2$, 0.8 mM $NADP^+$ and glucose 6-phosphate were used as substrate. The enzyme activity was determined by measuring the reduction of $NADP^+$ at 340 nm as previously described by Lupiáñez et al. [2]and Peragón et al. [50]. The change in absorbance at 340 nm was recorded and, after confirmation of no exogenous activity, the reaction started by the addition of substrate.

Glutathione S-transferase (GST; EC 2.5.1.18) activity was measured according to the method described by Habig et al.[51], using 1-chloro-2,4-dinitrobenzene as a substrate.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was determined using the method described by Flohe and Günzler [52], based on the oxidation of NADPH, which is used to regenerate the reduced glutathione (GSH) from oxidized glutathione (GS-SG) obtained by the action of glutathione peroxidase.

Glutathione reductase (GR, EC 1.8.1.7) was determined by the modified method of Carlberg and Mannervik [53]. We measured the decrease in absorbance produced by the oxidation of NADPH used by GR in the passage of oxidized glutathione (GS-SG) to reduced glutathione (GSH).

All enzyme activities (except SOD) are expressed as units or milliunits per milligram of soluble protein (specific activity). One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate per min under the above assay conditions.

Soluble protein concentration was determined using the method of Bradford, with bovine serum albumin used as a standard.

4.6. Statistical analysis

Data are showed as mean ± standard error mean (SEM). The statistical significance among different experimental groups was determined by one-way analysis of variance (ANOVA) test. When *F* values ($P<0.05$) were significant, means were compared using Tukey's HSD test. SPSS version 15.0 for Windows software package was used for statistical analysis.

5. Conclusions

In conclusion, the results obtained in the present study demonstrate that MA exerts a selective anti-proliferative effect against B16F10 cell line, whereas in A10 healthy cells, MA did not present cytotoxic effect. This natural compound prevent oxidative stress caused by H_2O_2 excess decreasing the ROS production levels. Moreover, depending on cell line, the anti-oxidant enzymatic response were different in presence H_2O_2 and without MA. So, in healthy A10 cells, all enzymes up-regulated their activity, but in B16F10 cells only SOD, GST and GPX increased it. In most cases, MA treatment restores the activities of enzymes to similar levels of the control group, highlighting that in A10 cells the doses of MA highest (IC_{50} and $2{\cdot}IC_{50}$) presented values below that control. Overall, these findings demonstrate the great anti-oxidant capacity of maslinic acid.

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Conflicts of Interest: The authors of this work declare that this research has been carried out in the absence of any commercial or financial relationship and, therefore, does not present any conflict of interest now or in the future.

References

1. Rodríguez-Rodríguez, R. Oleanolic acid and related triterpenoids from olives on vascular function: molecular mechanisms and therapeutic perspectives. *Curr. Med. Chem.* **2015,** *22*, 1414-1425.
2. Lupiáñez, J.A.; Adroher, F.J.; Vargas, A.M.; Osuna, A. Differential behaviour of glucose 6-phosphate dehydrogenase in two morphological forms of *Trypanosoma cruzi*. *Int. J. Biochem.* **1987,** *19*, 1085-1089.
3. Adroher, F.J.; Osuna, A.; Lupiáñez, J.A. Differential energetic metabolism during *Trypanosoma cruzi* differentiation. I: Citrate synthase, NADP-isocitrate and succinate dehydrogenases. *Arch. Biochem. Biophys.* **1988,** *267*, 252-261.
4. Adroher, F.J.; Osuna, A.; Lupiáñez, J.A. Differential energetic metabolism during *Trypanosoma cruzi* differentiation. II. Hexokinase, phosphofructokinase and pyruvate kinase. *Mol. Cell. Biochem.* **1990,** *94*, 71-82.
5. Peragón, J.; Barroso, J.B.; de la Higuera, M.; Lupiáñez, J.A. Relationship between growth and protein turnover rates and nucleic acids in the liver of rainbow trout (*Oncorhynchus mykiss*) during development. *Can. J. Fish. Aquat. Sci.* **1998,** *55*, 649-657.
6. Peragón, J.; Barroso, J.B.; García-Salguero, L.; Aranda, F.; de la Higuera, M.; Lupiáñez, J.A. Selective changes in the protein-turnover rates and nature of growth induced in trout liver by long-term starvation followed by re-feeding. *Mol. Cell. Biochem.* **1999,** *201*, 1-10.
7. Peragón, J.; Barroso, J.B.; García-Salguero, L.; de la Higuera, M.; Lupiáñez, J.A. Dietary alterations in protein, carbohydrates and fat increase liver protein-turnover rate and decrease overall growth rate in the rainbow trout (*Oncorhynchus mykiss*). *Mol. Cell. Biochem.* **2000,** *209*, 97-104.
8. Peragón, J.; Barroso, J.B.; García-Salguero, L.; de la Higuera, M.; Lupiáñez, J.A. Dietary-protein effects on growth and fractional protein-synthesis and degradation rates in liver and white muscle of rainbow-trout (*Oncorhynchus mykiss*). *Aquaculture* **1994,** *124*, 35-46.
9. Barroso, J.B.; Peragón, J.; García-Salguero, L.; de la Higuera, M.; Lupiáñez, J.A. Carbohydrate deprivation reduces NADPH-production in fish liver but not in adipose tissue. *Int. J. Biochem. Cell Biol.* **2001,** *33*, 785-796.
10. Peragón, J.; Barroso, J.B.; García-Salguero, L.; de la Higuera, M.; Lupiáñez, J.A. Growth, protein-turnover rates and nucleic-acid concentrations in the white muscle of rainbow trout during development. *Int. J. Biochem. Cell Biol.* **2001,** *33*, 1227-1238.
11. Sánchez-Muros, M.J.; García-Rejón, L.; Lupiáñez, J.A.; de la Higuera, M. Long-term nutritional effects on the primary liver and kidney metabolism in rainbow trout (*Oncorhynchus mykiss*). II. Adaptive response of glucose 6-phosphate dehydrogenase activity to high-carbohydrate/low-protein and high-fat/on-carbohydrate diets. *Aquacult. Nutr.* **1996,** *2*, 193-200.
12. Nóbrega-Pereira, S.; Fernández-Marcos, P.J.; Brioche, T.; Gómez-Cabrera, M.C.; Salvador-Pascual, A.; Flores, J.M.; Vina, J.; Serrano, M. G6PD protects from oxidative damage and improves healthspan in mice. *Nat. Commun.* **2016,** *7*, 10894.
13. Brennan, J.P.; Southworth, R.; Medina, R.A.; Davidson, S.M.; Duchen, M.R.; Shattock, M.J. Mitochondrial uncoupling, with low concentration FCCP, induces ROS-dependent cardioprotection independent of KATP channel activation. *Cardiovas. Res.* **2006,** *72*, 313-321.

14. Kurze, A.K.; Buhs, S.; Eggert, D.; Oliveira-Ferrer, L.; Muller, V.; Niendorf, A.; Wagener, C.; Nollau, P. Immature O-glycans recognized by the macrophage glycoreceptor CLEC10A (MGL) are induced by 4-hydroxy-tamoxifen, oxidative stress and DNA-damage in breast cancer cells. *Cell Commun. Signal.* **2019,** *17*, 107.

15. Mokhtari, K.; Rufino-Palomares, E.E.; Pérez-Jiménez, A.; Reyes-Zurita, F.J.; Figuera, C.; García-Salguero, L.; Medina, P.P.; Peragón, J.; Lupiáñez, J.A. Maslinic acid, a triterpene from olive, affects the antioxidant and mitochondrial status of B16F10 melanoma cells grown under stressful conditions. *Evid-Based Compl. Alt.* **2015**, ID 272457.

16. Ahmad, K.; Hafeez, Z.B.; Bhat, A.R.; Rizvi, M.A.; Thakur, S.C.; Azam, A.; Athar, F. Antioxidant and apoptotic effects of *Callistemon lanceolatus* leaves and their compounds against human cancer cells. *Biomed. Pharmacother.* **2018,** *106*, 1195-1209.

17. Sen, C.K. Cellular thiols and redox-regulated signal transduction. *Curr. Top. Cell. Regul.* **2000,** *36*, 1-30.

18. Zhang, Y.S.; Ning, Z.X.; Yang, S.Z.; Wu, H. Antioxidation properties and mechanism of action of dihydromyricetin from *Ampelopsis grossedentata*. *Acta Pharm. Sin.* **2003,** *38*, 241-244.

19. Yang, W.F.; Zhao, W.L. Determination of ginsenosides Re, Rb1 in Panax quinquefolius by micellar electrokinetic chromatography. *China J. Chin. Mat. Med.* **2003,** *28*, 1135-1137.

20. Reyes-Zurita, F.J.; Medina-O'Donnell, M.; Ferrer-Martín, R.M.; Rufino-Palomares, E.E.; Martín-Fonseca, S.; Rivas, F.; Martínez, A.; García-Granados, A.; Pérez-Jiménez, A.; García-Salguero, L.; Peragón, J.; Mokhtari, K.; Medina, P.P.; Parra, A.; Lupiáñez, J.A. The oleanolic acid derivative, 3-O-succinyl-28-O-benzyl oleanolate, induces apoptosis in B16-F10 melanoma cells via the mitochondrial apoptotic pathway. *Rsc. Adv.* **2016,** *6*, 93590-93601.

21. Reyes-Zurita, F.J.; Rufino-Palomares, E.E.; Lupiáñez, J.A.; Cascante, M. Maslinic acid, a natural triterpene from *Olea europaea* L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. *Cancer Lett.* **2009,** *273*, 44-54.

22. Reyes, F.J.; Centelles, J.J.; Lupiáñez, J.A.; Cascante, M. (2Alpha,3beta)-2,3-dihydroxyolean-12-en-28-oic acid, a new natural triterpene from *Olea europea*, induces caspase dependent apoptosis selectively in colon adenocarcinoma cells. *FEBS Lett.* **2006,** *580*, 6302-6310.

23. Rufino-Palomares, E.E.; Pérez-Jiménez, A.; Reyes-Zurita, F.J.; García-Salguero, L.; Mokhtari, K.; Herrera-Merchán, A.; Medina, P.P.; Peragón, J.; Lupiáñez, J.A. Anti-cancer and anti-angiogenic properties of various natural pentacyclic tri-terpenoids and some of their chemical derivatives. *Curr. Org. Chem.* **2015,** *19*, 919-947.

24. Siewert, B.; Csuk, R. Membrane damaging activity of a maslinic acid analog. *Eur. J. Med. Chem.* **2014,** *74*, 1-6.

25. Montilla, M.P.; Agil, A.; Navarro, M.C.; Jiménez, M.I.; García-Granados, A.; Parra, A.; Cabo, M.M. Antioxidant activity of maslinic acid, a triterpene derivative obtained from *Olea europaea*. *Planta Med.* **2003,** *69*, 472-474.

26. Barroso, J.B.; García-Salguero, L.; Peragón, J.; de la Higuera, M.; Lupiáñez, J.A. The influence of dietary-protein on the kinetics of NADPH production systems in various tissues of rainbow-trout (*Oncorhynchus mykiss*). *Aquaculture* **1994,** *124*, 47-59.

27. Allouche, Y.; Warleta, F.; Campos, M.; Sánchez-Quesada, C.; Uceda, M.; Beltrán, G.; Gaforio, J.J. Antioxidant, antiproliferative, and pro-apoptotic capacities of pentacyclic triterpenes found in the skin of olives on MCF-7 human breast cancer cells and their effects on DNA damage. *J. Agric. Food Chem.* **2011,** *59*, 121-130.

28. Xie, B.J.; Yang, S.P.; Chen, H.D.; Yue, J.M. Agladupols A-E, triterpenoids from *Aglaia duperreana*. *J. Nat. Prod*. **2007,** *70*, 1532-1535.
29. Chen, J.C.; Zhang, G.H.; Zhang, Z.Q.; Qiu, M.H.; Zheng, Y.T.; Yang, L.M.; Yu, K.B. Octanorcucurbitane and cucurbitane triterpenoids from the tubers of *Hemsleya endecaphylla* with HIV-1 inhibitory activity. *J. Nat. Prod*. **2008,** *71*, 153-155.
30. Reyes-Zurita, F.J.; Rufino-Palomares, E.E.; García-Salguero, L.; Peragón, J.; Medina, P.P.; Parra, A.; Cascante, M.; Lupiáñez, J.A. Maslinic acid, a natural Tj triterpene, induces a death receptor-mediated apoptotic mechanism in Caco-2 p53-deficient colon adenocarcinoma cells. *PloS one* **2016,** *11*, e0146178.
31. Rufino-Palomares, E.E.; Reyes-Zurita, F.J.; García-Salguero, L.; Mokhtari, K.; Medina, P.P.; Lupiáñez, J. A.; Peragón, J. Maslinic acid, a triterpenic anti-tumoural agent, interferes with cytoskeleton protein expression in HT29 human colon-cancer cells. *J. Proteomics* **2013,** *83*, 15-25.
32. Kim, Y.K.; Yoon, S.K.; Ryu, S.Y. Cytotoxic triterpenes from stem bark of *Physocarpus intermedius*. *Planta Med*. **2000,** *66*, 485-486.
33. Reyes-Zurita, F.J.; Pachón-Pena, G.; Lizarraga, D.; Rufino-Palomares, E.E.; Cascante, M.; Lupiáñez, J.A. The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism. *BMC Cancer* **2011,** *11*, 154.
34. Sánchez-Tena, S.; Reyes-Zurita, F.J.; Díaz-Moralli, S.; Vinardell, M.P.; Reed, M.; García-García, F.; Dopazo, J.; Lupiáñez, J.A.; Gunther, U.; Cascante, M. Maslinic acid-enriched diet decreases intestinal tumorigenesis in Apc(Min/+) mice through transcriptomic and metabolomic reprogramming. *PloS One* **2013,** *8*, e59392.
35. Reyes-Zurita, F.J.; Rufino-Palomares, E.E.; Medina, P.P.; García-Salguero, L.; Peragón, J.; Cascante, M.; Lupiáñez, J.A. Antitumour activity on extrinsic apoptotic targets of the triterpenoid maslinic acid in p53-deficient Caco-2 adenocarcinoma cells. *Biochimie* **2013,** *95*, 2157-2167.
36. Zhang, S.; Ding, D.; Zhang, X.; Shan, L.; Liu, Z. Maslinic acid induced apoptosis in bladder cancer cells through activating p38 MAPK signaling pathway. *Mol. Cell. Biochem.* **2014,** *392*, 281-287.
37. Medina, I.; Lois, S.; Lizarraga, D.; Pazos, M.; Tourino, S.; Cascante, M.; Torres, J. L. Functional fatty fish supplemented with grape procyanidins. Antioxidant and proapoptotic properties on colon cell lines. *J. Agric. Food Chem.* **2006,** *54*, 3598-3603.
38. Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol.* **2003,** *41*, 753-758.
39. Choi, B.S.; Kim, H.; Lee, H.J.; Sapkota, K.; Park, S.E.; Kim, S.; Kim, S.J. Celastrol from 'Thunder God Vine' protects SH-SY5Y cells through the preservation of mitochondrial function and inhibition of p38 MAPK in a rotenone model of Parkinson's disease. *Neurochem. Res.* **2014,** *39*, 84-96.
40. Rafatian, G.; Khodagholi, F.; Farimani, M.M.; Abraki, S. B.; Gardaneh, M. Increase of autophagy and attenuation of apoptosis by Salvigenin promote survival of SH-SY5Y cells following treatment with H_2O_2. *Mol. Cell. Biochem.* **2012,** *371*, 9-22.
41. Baricevic, D.; Sosa, S.; Della Loggia, R.; Tubaro, A.; Simonovska, B.; Krasna, A.; Zupancic, A., Topical anti-inflammatory activity of *Salvia officinalis* L. leaves: the relevance of ursolic acid. *J. Ethnopharmacol.* **2001,** *75*, 125-132.
42. Yang, Z.G.; Li, H.R.; Wang, L.Y.; Li, Y.H.; Lu, S.G.; Wen, X.F.; Wang, J.; Daikonya, A.; Kitanaka, S. Triterpenoids from *Hippophae rhamnoides* L. and their nitric oxide production-inhibitory and DPPH radical-scavenging activities. *Chem. Pharm. Bull.* **2007,** *55*, 15-18.

43. Kirkman, H.N.; Rolfo, M.; Ferraris, A.M.; Gaetani, G.F. Mechanisms of protection of catalase by NADPH - Kinetics and stoichiometry. *J. Biol. Chem.* **1999,** 274, 13908-13914.
44. Pérez-Jiménez, A.; Abellán, E.; Arizcun, M.; Cardenete, G.; Morales, A.E.; Hidalgo, M.C. Dietary carbohydrates improve oxidative status of common dentex (*Dentex dentex*) juveniles, a carnivorous fish species. *Comp. Biochem. Physiol. A* **2017,** *203*, 17-23.
45. Wang, W.; Xu, Z.; Yang, M.; Liu, R.; Wang, W.; Liu, P.; Guo, D. Structural determination of seven new triterpenoids from *Kadsura heteroclita* by NMR techniques. *Magn. Reson. Chem.* **2007,** *45*, 522-526.
46. Rao, M.S.; Subbarao, V. Effect of dexamethasone on ciprofibrate-induced cell proliferation and peroxisome proliferation. *Fundam. Appl. Toxicol.* **1997,** *35*, 78-83.
47. Rothe, G.; Oser, A.; Valet, G. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. *Die Naturwissenschaften* **1988,** *75*, 354-355.
48. McCord, J.M.; Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **1969,** *244*, 6049-6055.
49. Aebi, H. Catalase in vitro. *Methods Enzymol.* **1984,** *105*, 121-126.
50. Peragón, J.; Aranda, F.; García-Salguero, L.; Corpas, F.J.; Lupiáñez, J.A. Stimulation of rat-kidney hexose-monophosphate shunt dehydrogenase-activity by chronic metabolic-acidosis. *Biochem. Int.* **1989,** *18*, 1041-1050.
51. Habig, W.H.; Pabst, M.J.; Jakoby, W.B. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J. Bio. Chem.* **1974,** *249*, 7130-7139.
52. Flohe, L.; Gunzler, W.A. Assays of glutathione peroxidase. *Methods Enzymol.* **1984,** *105*, 114-121.
53. Carlberg, I.; Mannervik, B. Purification by affinity chromatography of yeast glutathione reductase, the enzyme responsible for the NADPH-dependent reduction of the mixed disulfide of coenzyme A and glutathione. *Biochim. Biophys. Acta* **1977,** *484*, 268-274.