Free Radical Research, March 2006; 40(3): 295–302

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Supressive effect of maslinic acid from pomace olive oil on oxidative stress and cytokine production in stimulated murine macrophages

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Accepted by Professor H. E. Poulsen

(Received 29 June 2005; in revised form 10 November 2005)

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Abstract

The pentacyclic triterpene maslinic acid (MA) is a natural compound present in the non glyceride fraction of pomace olive oil, also called *orujo* olive oil. This compound has previously demonstrated antioxidant properties against lipid peroxidation *in vitro*, but its effects on reactive oxygen and nitrogen-derived species and pro-inflammatory cytokines generated by a cell system have not yet been investigated. In this study, we have tested the effect of MA upon oxidative stress and cytokine production using peritoneal murine macrophages. MA significantly inhibited the enhanced production of nitric oxide (NO) induced by lypopolysaccharide (LPS) when it was measured by the nitrite production with an inhibitory concentration 50% value (IC_{50}) of 25.4 μM. This inhibiting effect seems to be consequence of an action at the level of the LPS-induction of the inducible nitric oxide synthethase (iNOS) gene enzyme expression rather than to a direct inhibitory action on enzyme activity. The secretion of the inflammatory cytokines interleukine-6 and TNF-α from LPS-stimulated murine macrophages was also significantly reduced ($p < 0.05$ and 0.01) by 50 and 100 μM of MA. In addition, reactive oxygen species were measured after stimulation with phorbol-12-myristate-13-acetate (PMA). Thus, pre-treatment with MA reduced the generation of hydrogen peroxide from stimulated macrophages in a dose-dependent manner (IC_{50}: 43.6 μM) as assayed by the oxidation of the peroxidase enzyme. However, no inhibitory effect on superoxide release, measured by the reduction of ferricytochrome *c*, was observed after the pretreatment with MA in the culture medium.

These results suggest a potential biopharmaceutical use of this hydroxy-pentacyclic triterpene derivative, present in *orujo* olive oil, on preventing oxidative stress and pro-inflammatory cytokine generation.

Keywords: *Maslinic acid, pentacyclic triterpene, pomace olive oil, murine macrophages, oxidative stress, nitric oxide, interleukine*

Abbreviations: *MA, maslinic acid; NO, nitric oxide; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; SNP, sodium nitroprusside; PMA, phorbol-12-myristate-13-acetate*

Introduction

There is growing interest in identifying components in foods, such as vegetables, red wine and olive oil, which could contribute to the well established beneficial health effects of the Mediterranean diet [1,2]. The concept that oxidative stress plays a key role in diseases development has led to the proposal that health might be improved by increased dietary intake of antioxidants such as those present in the foods mentioned above. In the case of the minor components of olive oil, different authors [3,4] and ourselves [5,6] have demonstrated antioxidant and antiinflammatory properties of phenolic compounds present in the polar fraction of this oil. Besides, olive

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500467935

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oil also contains other minor components of a non polar nature in its unsaponifiable or non glyceride matter. The composition and concentration of the components of this fraction are complex and specific for each type of vegetable oil. The fraction comprises of several groups of compounds: phospholipids, hydrocarbons, sterols, triterpenes, tocopherols, chlorophylls and flavour compounds [7,8]. We have previously reported a potent anti-inflammatory action produced by triterpenic and steroidal compounds isolated from the unsaponifiable fraction of virgin olive oil [9]. More recently, we have detected a favorable effect of the unsaponifiable fraction of virgin olive oil on the endothelial function by reducing pro-inflammatory eicosanoid generation [10].

Pomace olive oil, also called *orujo* olive oil, is a blend of refined-pomace oil and virgin olive oil. *Orujo* olive oil fits for human consumption and is ordinarily consumed in Spain and other Mediterranean countries. Pomace is the solid residual derived after first olive pressing or centrifugation and is constituted of a residual of olives, skin and seeds. This waste is a good source of triterpenic and steroidal compounds. Due to this, these compounds are specifically found at a higher concentration in pomace olive oil than in virgin olive oil [11]. We have recently demonstrated that pomace olive oil is as effective as olive oil in preventing lipid peroxidation. This effect might partly be explained by its triterpenoids erythrodiol and oleanolic acid content [12].

Triterpenes have been described as effective anti-inflammatory agents and are recognised as the active principles of several therapeutically used medicinal plants [13,14]. One of the best known triterpenes found in pomace olive oil is oleanolic acid (3β-hydroxyolean-12-en-28-oic acid). This compound has been reported to effectively inhibit cyclo-oxygenase and 5-lipoxygenase enzymes from the arachidonate cascade [15]. Other biological and pharmacological properties have been summarised by Liu [16]. From our experience, an endothelium-dependent vasorelaxation in rat aorta was evoked by erythrodiol and oleanolic acid. This suggests a nitric oxide (NO)-related mechanism [17,18].

Maslinic acid (MA; 2-α,3β-dihydroxyolean-12-en-28-oic acid) is another pentacyclic triterpene from the oleanane family present in pomace olive oil that merits further investigation. This compound, whose chemical structure differs from oleanolic acid by the presence of an additional hydroxyl group at the 2 carbon position (Figure 1), has demonstrated its antioxidant properties by decreasing the susceptibility of plasma and hepatocyte membranes to lipid peroxidation [19]. This compound has also shown inhibitory activity against HIV-1 protease [20,21].

Macrophages play a significant role in host defence mechanism. Reactive oxygen and nitrogen species, such as superoxide anion, peroxide, hydroxy radical and nitrite radical are generated when these cells are activated. They can act as mediators of inflammation

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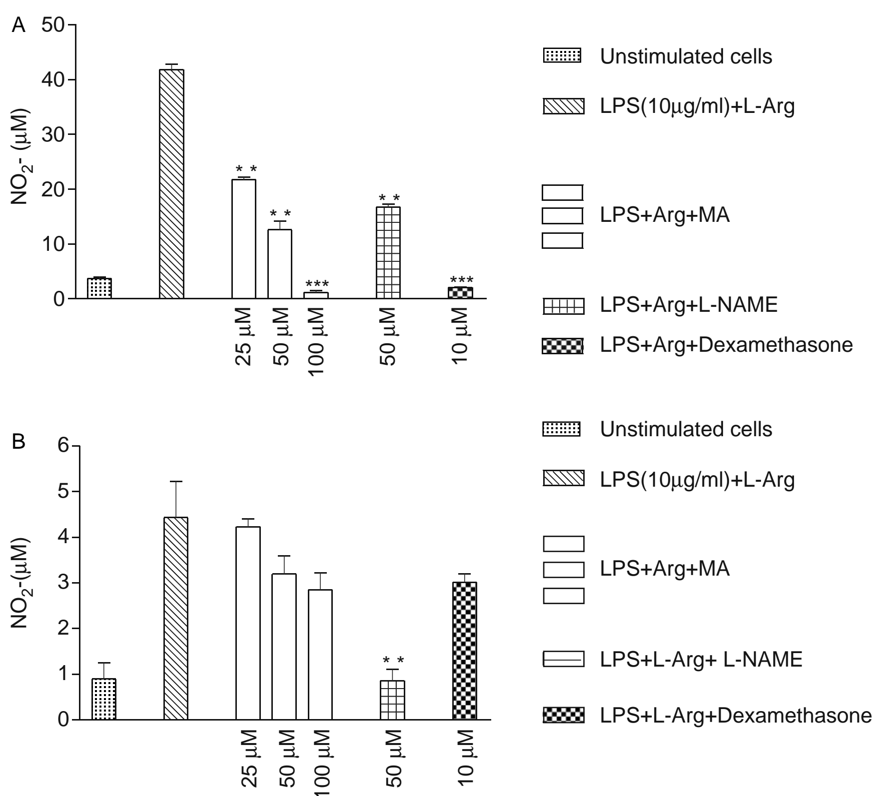

Figure 1. Chemical structures of pentacyclic triterpenes from pomace olive oil.

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when overproduced [22,23]. In addition, overproduction of pro-inflammatory cytokines, such as interleukine-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), plays a key role in the onset and progress of inflammatory diseases [24–26]. Compounds effective in supressing macrophages response to activation can significantly reduced inflammation processes. The aim of this study was to determine whether MA plays a protective effect against oxidative stress and pro-inflammatory cytokine secretion in stimulated murine macrophages.

Materials and methods

Experimental animals

Male Swiss albino mice weighing 20–25 g were used. The animals were supplied by the "Centro de Instrumentación Científica" from the University of Granada (Spain). All animals were maintained in suitable nutritional and environmental conditions throughout the experiments. All experiments were performed according to the European Union guidelines for the ethical treatment of animals.

Chemicals

The hydroxy pentacyclic triterpene derivative MA was obtained by the patented method from García-Granados et al. from olive-pressing residues [27].

RPMI 1640 medium were from Biochrom AG (Berlín). Fetal bovine serum, antibiotics, L-glutamine, tioglycolate, 3-(4,5-dimethylthiazol-2-yl)-2,diphenyl-tetrazolium bromide MTT, phenol red, sodium nitroprusside (SNP), N-(1-Naphthyl) ethenediamine-2HCl and sulphanilamine, sodium nitrite, phorbol-12-myristate-13-acetate (PMA), lipopolysaccharide (LPS), superoxide dismutase (SOD), cytochrome *c*, catalase, horseradish peroxidase, L-G-Nitroarginine methyl esther (L-NAME), dexamethasone, Hanks' balanced salt solution (HBSS) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Química (Madrid). TNF-α and IL-6 ELISA kits were purchased from Endogen Perbio Science (France).

Isolation and culture of murine peritoneal macrophages

The mice were used to obtain highly purified peritoneal macrophages that were isolated and cultured as previously described [28]. Cells were harvested by peritoneal lavage 4 days after a intraperitoneal injection of 1 ml of 10% thioglycolate broth. Cells were then resuspended in culture RPMI 1640 medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and incubated at 37°C in a 5% CO_2 humidified incubator for 2 h. The adherent cells were used to perform the experiments. The mitocondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) to formazan was used to assess the possible cytotoxic effects of the test compound on murine peritoneal macrophages [29].

Nitrite (NO_2^-), IL-6, and TNF-α production in murine peritoneal macrophages (induction phase)

Murine peritoneal macrophages (10^6 cells/well) were cultured in 24-well plates, stimulated with LPS (final concentration 10 μg/ml) and co-incubated with different doses of MA (25, 50 and 100 μM) or L-NAME (50 μM) or dexamethasone (10 μM) at 37°C for 24 h. As an indicator of NO production, nitrite concentration was measured in the supernatants. Equal volumes of culture supernatants and Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% *N*-(naphthylethylenediamine-HC) were mixed and the absorbance was read at 450 nm as described elsewhere [30]. The amount of nitrite was obtained by a extrapolation from a standard curve with sodium nitrite.

IL-6 and TNF-α were measured in culture supernatants by inmmunoassay (kits from Endogen Perbio Science, France).

iNOS activity in murine peritoneal macrophages (post-induction phase)

In a different set of experiments, a cultured medium of 24 h LPS-stimulated macrophages (10^6/well) in the absence of the test compound was removed. A new medium, supplemented with L-arginine (0.5 mM) and MA or L-NAME (50 μM), was added to the plate and incubated for further 2 h as described elsewhere [31]. At 2 h supernatants were collected and nitrite was measured by the Griess reaction as described above.

Assay of scavenging of NO radicals released by sodium nitroprusside

This was carried out in order to investigate whether MA is able to remove NO by scavenging it. Different doses of MA were added to 1 ml of SNP (5 mM) in PBS and incubated at 25°C for 2 h. Curcumin at 50 μM was used as a reference compound. After incubation, the concentration of nitrite was measured by the Griess method as described above. A negative control, by using a solution of sodium nitrite (instead of SNP) was carried out.

Superoxide and hydrogen peroxide measurements

Superoxide secretion by macrophages in response to PMA was measured by the oxidation of reduced cytochrome *c* [32]. Macrophages (10^6 cells/well), once adhered to the plate, were preincubated in 1000 μl HBSS containing 2% glucose (w/v) and cytochrome *c* (80 μM) with doses of the test compound or SOD

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(250 μg/ml) at 37°C for 30 min. Stimulation was performed adding 10μl PMA to give a final concentration of 1 μM. After 2 h the medium was removed and the absorbance was read at 550 nm. Superoxide concentrations were calculated using the molar extinction coefficient of oxidized cytochrome *c* ($2.1 \times 10^4 M^{-1} cm^{-1}$).

Hydrogen peroxide secretion by macrophages in response to stimulation by PMA was measured by oxidation of phenol red in the presence of peroxidase [33]. Macrophages (10^6 cells/well), once adhered to the plate, were preincubated in 1000 μl HBSS containing 0.1% glucose (w/v), 10 μl phenol red (28 mM) and 10 μl horseradish peroxidase (Type II from Sigma) (50 μg/mL) with doses of the test compound or catalase (oxidoreductase EC 1.11.1.6.; at 1250 u/ml) at 37°C for 30 min. The stimulation was performed adding 10μl PMA to give a final concentration of 1 μM. After 2 h the medium was removed, 10 μl NaOH 1 N was added, and the absorbance was read at 610 nm. Hydrogen peroxide concentrations were calculated by comparision to a standard curve.

Assay of scavenging of H_2O_2

This was carried out in order to investigate whether MA was able to remove H_2O_2 by scavenging it. MA scavenger activity was measured by adding 10 μl of the test compound to 100 μl of H_2O_2 and 900 μl of buffer HBSS. After incubating the mixture for 30 min at room temperature, 50 μl of a guaiacol solution (0.2% v/v in distilled water), and 10 μl of horseradish peroxidase (5 mg/ml) were added. After 10 min of incubation the absorbance was read at 450 nm in a Labsystem Multiscan EX Prymary EIA V.2.1.

Statistical analysis

Statistical evaluation included one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) were taken as significant. Results are shown as mean ± S.E.M. for *n* experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least three significant concentrations.

Results

Effects of maslinic acid on nitrite, interleukine-6 and TNF-α production in murine peritoneal macrophages

MA significantly inhibited nitrite production in a dose-dependent manner (Figure 2(A)). The calculated

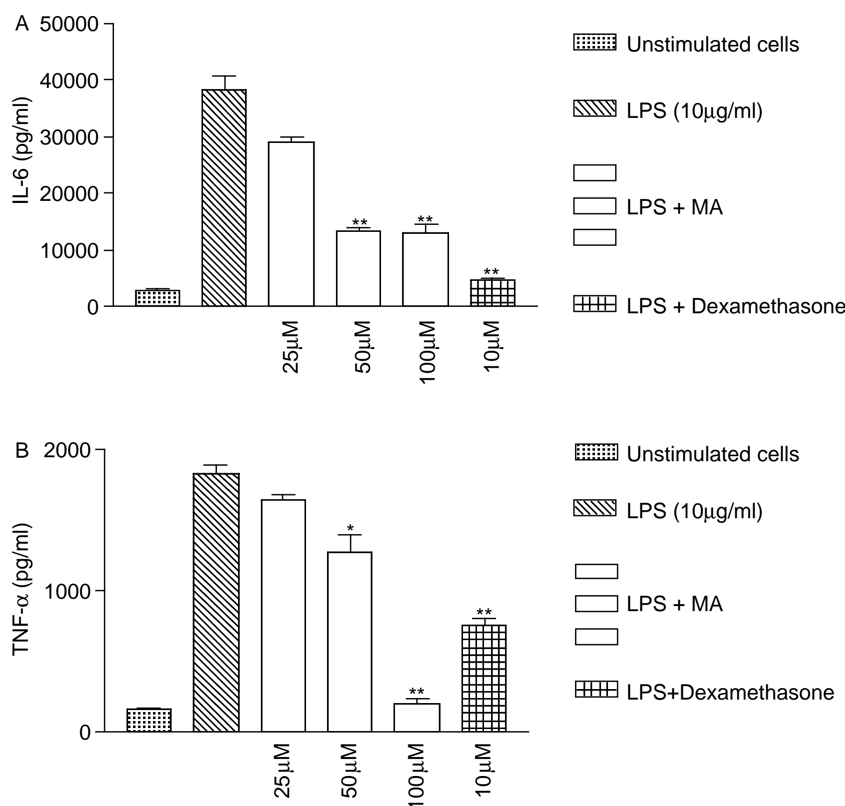

Figure 2. Effect of MA on NO production by murine macrophages. (A) Induction phase: (10^6 cells/ml) macrophages were incubated with LPS (10 μg/ml) in the presence of several concentrations of MA or L-NAME and dexamethasone for 24 h. (B) Post-induction phase: (10^6 cells/ml) macrophages were incubated with LPS (10 μg/ml) in the absence of the test compound for 24 h. After washing the cells, MA was added and macrophages incubated for 2 h in the presence of L-arginine (0.5 mM). Each value represents the mean ± S.E.M. for three triplicate experiments. $p < 0.01$ (**), $p < 0.001$ (***) versus LPS-stimulated cells.

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IC_{50} was 25.4 μM. A concentration of 50 μM of MA produced a similar effect to that of the reference inhibitor L-NAME. A dose of 100 μM completely suppressed the production of nitrite. It should be taken into account, however, that at this latter dose MA showed a cytotoxicity of about 30% when assessed by mitochondrial reduction of MTT after 24 h treatment (data not shown). At lower doses no cytotoxic effects were found for MA.

In order to determine whether the inhibition of nitrite production was either due to an interference with the enzyme induction by LPS or due to a direct action of the compound on NOS activity, MA was incubated with cells after the induction of iNOS by LPS for 24 h. After the cells were washed and cultured with a new medium supplemented with L-arginine (0.5 mM) for further 2 h incubation. MA, which produced a significant reduction in nitrite production in the induction phase, did not significantly affect nitrite content in the post-induction phase, whereas the selective L-NAME, a known inhibitor of NOS activity, caused a total reduction of nitrite. The reference control, the steroid anti-inflammatory dexamethasone, significantly inhibited production of NO induced by LPS, but not NOS activity as has also been previously described [31] (Figure 2(B)).

IL-6 and TNF-α production were measured in the supernatants from the first experiment. A significant reduction ($p < 0.05$ and 0.01) on the cytokine generation was observed with both MA concentrations of 50 and 100 μM compared to the control group of macrophages stimulated with LPS (10 μg/ml) but in absence of MA. We did not observe, however, a clear dose-dependent inhibition relationship (Figure 3 (A) and (B)).

Scavenging of NO by maslinic acid

The NO-radical-scavenging activity of MA was evaluated in experiments using SNP as NO donor. MA showed only a slight scavenging effect at 50 and 100 μM (Table I).

Effect of MA on superoxide and hydrogen peroxide production

Preincubation of murine peritoneal macrophages with MA did not affect superoxide production induced by stimulating the cells with PMA (1 μM) when assayed by the reduction of ferricytochrome *c* (Figure 4(A)). This indicates that this compound neither inhibits the NADPH-oxidases responsible for the oxidative burst process nor scavenges the superoxide radical.

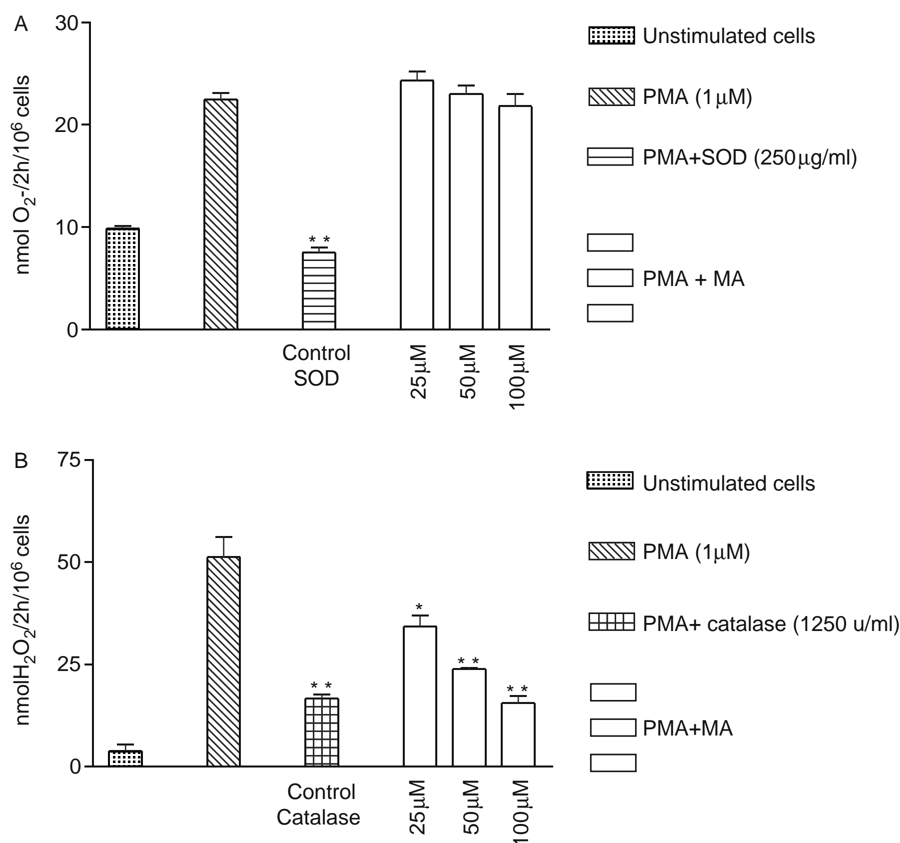

Figure 3. Effect of MA on IL-6 and TNF-α production by (10^6 cells/ml) murine macrophages once they were stimulated with LPS (10 μg/ml) in the presence of several concentrations of MA or dexamethasone for 24 h. Each value represents the mean ± S.E.M. for two triplicate experiments. $P < 0.05$ (*), $p < 0.01$ (**) versus LPS-stimulated cells.

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Table I. Effect of maslinic acid on scavenging of NO radicals released by SNP.

	NO scavenging (% of control)
Control	
Maslinic acid	
25 μM	11.70 ± 2.5
50 μM	20.60 ± 1.2*
100 μM	21.04 ± 2.1*
Curcumin	
50 μM	62.03 ± 3.5**

Results are expressed as mean percentage inhibition with respect to the control ± S.E.M. (*$p < 0.05$ **$p < 0.01$)

As expected, SOD substantially reduced the amount of detectable superoxide.

Nevertheless, MA was able to reduce the formation of hydrogen peroxide, in a similar way the catalase enzyme did, at a final concentration of 1250 U/ml (Figure 4(B)). It was observed that MA inhibited H_2O_2 release in a dose dependent-manner with a IC_{50} of 43.6 μM. Furthermore, we could test that this action was not due to a direct scavenging action of H_2O_2 by MA when it was measured by the guaiacol and peroxidase enzyme reaction (data not shown).

Discussion

MA is a natural pentacyclic triterpene present in olives and in the unsaponifiable fractions of pomace olive oil, obtained from olive wastes. This compound has been reported to possess antioxidant properties by preventing lipid peroxidation [19]. In this study, we have demonstrated that MA can decrease oxidative stress modulating the release of oxygen reactive species, such as NO and H_2O_2, and the pro-inflammatory cytokines IL-6 and TNF-α, in stimulated murine peritoneal macrophages.

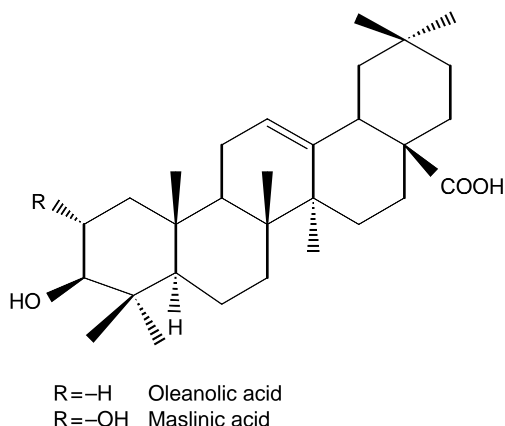

Figure 4. Effect of MA on superoxide and hydrogen peroxide release by murine peritoneal macrophage stimulated with PMA (1 μM) for 2 h. (A) Superoxide measurements performed by cytochrome *c* assay. (B) Hydrogen peroxide measurements performed by the oxidation of phenol red in the presence of peroxidase enzyme. Each value represents the mean ± S.E.M. for three triplicate experiments. $P < 0.05$ (*), $p < 0.01$ (**) versus PMA-stimulated cells.

NO is involved in phagocytosis at a physiological level. NO, however, can enhance inflammation and tissue injury when it is produced in a large amount [34]. LPS treatment to cells significantly elevated NO generation by 36 times more than the LPS untreated negative control. This has been well-documented by several researchers [35,36]. MA supresses NO production in a dose-dependent manner with an IC_{50} in the micromolar range (25.4 μM). Data described above suggest that the inhibition of NO production by MA in murine macrophages might occur at the level of the LPS-induced iNOS enzyme gene expression rather than at the NOS enzyme activity. The behaviour of MA being more similar to that of the reference compound dexamethasone than to L-NAME.

The onset of the NO production induced by LPS requires the activation of the nuclear factor (NF)-κB for the susequent iNOS mRNA expression [37], The effect of MA on iNOS could be via blocking NF-kappaB activation, as this action has been extensively reported for other triterpene compounds as well as for dexamethasone [38–40]. Further studies are required to confirm this hypothesis. It has been also reported that synthetic modifications on the structure of natural oleanane triterpenoids have resulted in potent inhibitors of the induction of iNOS in mouse macrophages [41–43]. In this sense, MA could be a possible model for developing anti-inflammatory agents.

It should be taken into account that other triterpenes, like oleanolic and ursolic acids, have been shown to enhance the production of NO and TNF-α as they are effective in upregulating iNOS and TNF-α expression through (NF)-κB transactivation in resting, not stimulated, macrophages. This effect explains part of these triterpenes anti-tumoral mechanism of action [44, 45].

Reactive molecules as superoxide, hydrogen peroxide and NO are produced from macrophages following exposure to LPS or PMA. The enzyme responsible for the production of O_2^- and H_2O_2 is a multi-component NADPH oxidase that requires assembly at the plasma membrane. Although, some triterpenes have been proven to suppress superoxide generation, in this experiment the assayed compound MA failed to inhibit the production of this radical. This effect has been reported to be influenced by the type of stimulant used. In this sense, the triterpenes α-amyrin and uvaol can suppressed the superoxide generated by the peptide f-MLP or arachidonic acid but not that from cells stimulated by PMA [46].

Nevertheless, we observed that MA effectively reduced the H_2O_2 generation in the μM range (IC_{50}: 43.6 μM). This agrees with the results from *in vivo* experiments carried out by other authors where it has been described that, in a model of a hydrogen

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peroxide-induced inflammation in rat skin, some pentacyclic triterpenoids showed a potent anti-oedematous effect. This action did not appear to be dependent on the chemical structure of these compounds [47]. Given that it has been reported that H_2O_2 participates in the up-regulation of iNOS expression in peritoneal macrophages [48], it could be hypothesized this mechanism as being implicated in the reduction of the NO production by MA.

Cytokines belong to a group of proteins secreted by stimulated macrophages, which regulate host defence and cause inflammation. MA at doses of 50 and 100 μM could effectively inhibit the production of the pro-inflammatory cytokines TNF-α and IL-6 from the LPS-stimulated macrophages. Given that pro-inflammatory cytokines are able to stimulate the transcription of iNOS to enhance NO production [49], it is conceivable that the NO-inhibitory effect of MA may partly occur through suppressed production of these pro-inflammatory cytokines.

In summary, MA may attenuate intracellular oxidative stress by inhibiting NO and H_2O_2 production and reducing pro-inflammatory cytokines generation. These results suggest a potential biopharmaceutical use of this hydroxy-pentacyclic triterpene derivative present in *orujo* olive oil, the specific components of which deserve further investigation.

Acknowledgements

The authors thank Prof A. García-Granados from Department of Organic Chemistry, Faculty of Sciences, University of Granada by the supply of the compound MA. Ana Márquez thanks the grant BES-2003-1392 received from the CICYT. This work was supported by the following projects AGL2002-00195, AGL2005-00572 from the CICYT (Comisión Interministerial de Ciencia y Tecnología) and G03-140 (from Red FIS de investigación cooperativa) conceded to V. Ruíz-Gutiérrez.

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