Antioxidant and antiatherogenic activities of pentacyclic triterpenic diols and acids

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ABSTRACT

The present paper aimed to test the potential cardioprotective activity of four pentacyclic triterpenes, uvaol, erythrodiol, oleanolic acid and maslinic acid, widely distributed throughout the vegetable kingdom. For this purpose, their antioxidant and antithrombotic activities related to LDL particles have been in vitro evaluated. Results demonstrated that maslinic acid, uvaol and erythrodiol exert antiatherogenic effect while no effect was observed for oleanolic acid. Specifically, maslinic acid has shown the most potent dose-dependent antioxidant effect and did not have antithrombotic properties, whereas uvaol and erythrodiol exhibited both antioxidant and antithrombotic activities. In addition, antioxidant mechanisms of action were determined. While maslinic acid possesses dual activity acting as scavenger of free radicals and as copper chelator, uvaol is able to form a complex with copper and erythrodiol seems to behave as a retarder antioxidant. In conclusion, dietary triterpenes may exert a cardioprotective effect by different mechanisms of action related to antioxidant and antithrombotic activities.

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1. Introduction

Atherosclerosis and its thrombotic complications remain by far the most common causes of morbidity and mortality in the developed countries. Atherosclerosis is an oxidative, inflammatory and thrombotic disease of the arterial wall that is precipitated by elevated levels of low-density lipoprotein (LDL) cholesterol in the blood (Badimón et al., 2009). Several lines of evidences support the hypothesis that the oxidation of LDL may play a critical role in early state of atherosclerosis, while thrombosis is one of the latest fatal clinical consequences of this disease (Lusis, 2000).

Diet is an important tool for the prevention of cardiovascular diseases (Ferdowsian and Barnard, 2009). Triterpenes are widely available in dietary fruits and vegetables, and are major components in many medicinal plants used in Asian countries (Setzer and Setzer, 2003). Most of them are biosynthesized in plants by cascade cyclizations and rearrangements of the acyclic precursors squalene and 2,3-oxidosqualene (Abe, 2007), leading to tetra and pentacyclic triterpene skeleta. In the present work, we focus on four pentacyclic triterpenes, uvaol, erythrodiol, oleanolic acid and maslinic acid (Fig. 1) present in olive oil, mainly in the nonglyceride fraction of pomace oil, obtained from Olea europaea L., and other plants such as Eucalyptus globulus labill, Uva-ursi, Acacia senegal, Chrysanthemum morifolium, Malus domestica, Punica granatum, Syzygium aromaticum, Salvia officinalis, Ziziphus zizyphus (Gotfredsen, 2008; Duke, 1998).

Different properties have been reported for these compounds. Among them, antioxidant, mainly LDL protective antioxidant effect (Andrikopoulos et al., 2002; Wang et al., 2006), hypolipidemic (Somova et al., 2003a; Lin et al., 2009), vasorelaxant and anti-hypertensive (Rodríguez-Rodríguez et al., 2006; Somova et al., 2003b) and anti-inflammatory (Márquez-Martín et al., 2007) properties may contribute to their cardiovascular benefits. However the mechanisms of action of these pentacyclic triterpenes on LDL proatherogenic effect are still unknown. Therefore, we have focused the aim of the present study in the evaluation of the in vitro LDL-related antioxidant and antithrombotic effects of uvaol, erythrodiol, oleanolic acid and maslinic acid. In addition, we have tried to elucidate the underlying mechanisms implicated in their antioxidant effects.

2. Materials and methods

2.1. Materials and reagents

Uvaol, erythrodiol and oleanolic acid were purchased from Extrasynthese (Genay, France). Maslinic acid was provided by Dr. A. Garcia-Granados, Department of Organic Chemistry, University of Granada, Spain. Stock solutions of these com-
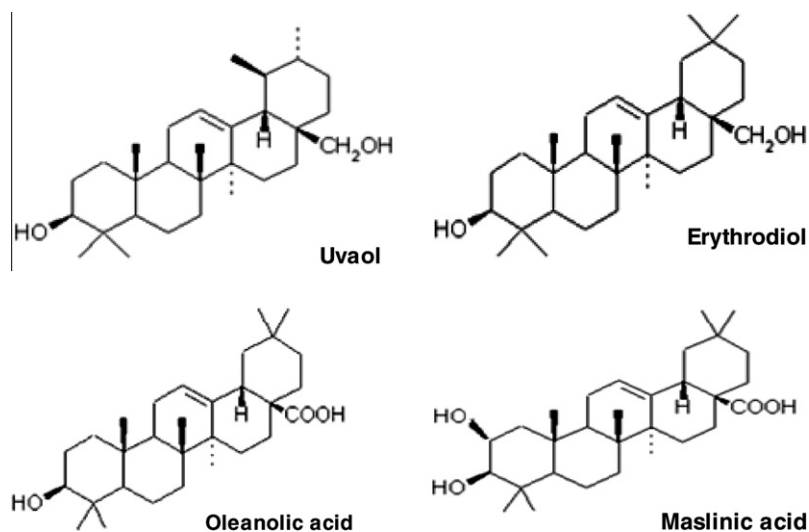
Fig. 1. Chemical structure of uvaol, erythrodiol, oleanolic acid and maslinic acid.

pounds were prepared in ethanol or DMSO and frozen at −20 °C until use. These solutions were then diluted in phosphate buffer saline (PBS) to reach the desired concentration for experiments.

2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8 tetramethylchroma-2-carboxylic acid (Trolox), α-tocopherol (vitamin E), bovine serum albumin (BSA), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), CuSO$_4$, EDTA and DMSO were obtained from Sigma Chemical Co. (Germany). PBS and Fluorescein (FL) disodium salt were obtained from Gibco, Invitrogen (UK). Ethanol, NaCl, HCl, KBr, Cl$_3$C$_2$O$_2$H and CaCl$_2$ were obtained from Panreac (Spain), while citric acid was purchased from Merck (Germany). Highly purified human plasma-derived prothrombin, coagulation factor V (FV) and activated factor X (FXa) were obtained from Diagnostica Stago (Asnières, France). These coagulations factors were reconstituted in distilled water. Purified thrombin was obtained from Calbiochem (USA) and was also reconstituted in distilled water. The chromogenic substrate for thrombin (S-2238) was purchased from Chromogenix-Instrumentation Laboratory SpA (Izasa, Spain).

2.2. In vitro LDL oxidation

2.2.1. LDL isolation

Fasting blood samples were collected by venipuncture in pre-chilled EDTA-coated vacuum tubes from healthy human volunteers. Immediately, plasma was separated by centrifugation at 1750g for 15 min at 4 °C. LDL was isolated by a single discontinuous-density gradient ultracentrifugation in a vertical rotor VTI-50 at 247,000g for 150 min at 4 °C, as described by Chung et al. (1981). The isolated LDL was exhaustively dialyzed overnight against 150 mmol/L NaCl, pH 7.4–7.6 at 4 °C. Fresh LDL isolates were used for oxidation and thrombin experiments in the following four days. LDL protein was measured using a bicinchoninic acid (BCA) reagent kit (Pierce Biotech, Rockford, USA).

2.2.2. LDL oxidation

LDL isolate was oxidized in PBS with 20 μM CuSO$_4$ at 37 °C in the absence (control containing the same proportion of ethanol than in test compounds) or presence of compounds (final concentration 10, 20 and 50 μM). LDL oxidation was monitored by measuring conjugated dienes and thiobarbituric acid reactive substances (TBARS) as markers of the early and later stages of LDL oxidation, respectively. α-Tocopherol was also assayed as a reference.

2.2.3. Conjugated dienes

LDL (20 μg/mL) was oxidized by the addition of 20 μM CuSO$_4$ at 37 °C. The formation of conjugated dienes was followed up immediately by monitoring the increase in the absorbance at 234 nm for 240 min in a temperature-controlled multidetection microplate reader (Synergy HT, BIO-TEK Instruments, VT, USA) (Mesa et al., 2008). Results were expressed as the lag phase (min), calculated from the course time graph (OD$_{234\,nm}$ and time) as the time point where the linear extrapolation of the propagation phase intercepts $y = y$ (t_0), and the rate of propagation determined by the maximum rise in absorbance at 234 nm per minute (ΔOD$_{234}$/min). Measurements were carried out at least in triplicate.

2.2.4. TBARS

LDL (200 μg/mL) was incubated with CuSO$_4$ (20 μM) at 37 °C for 16 h. After this period, oxidation was stopped by addition of EDTA and BHT at final concentration 20 mM and 40 mM, respectively. TBARS were determined at 532 nm in a Perkin Elmer

UV–VIS Lambda 40 spectrophotometer as previously reported by Mesa et al. (2008). Quantitative analysis was performed employing a 1,1,3,3-tetraetoxypropane calibration curve in the range of concentration 0.2–10 nM. Data were expressed as nmoles of malondialdehyde (MDA)/mg of LDL protein. Analyses were performed in triplicate.

2.3. ORAC assay

The peroxyl radical scavenging activity of the tested triterpenes was measured by ORAC$_{FL}$ assay as previously described by Prior et al. (2003) with some modifications. Trolox was used as a control antioxidant standard. Tested compounds were dissolved in DMSO and then diluted with PBS. The same amount of DMSO was also added to the blank and did not affect the ORAC assay. The assay was carried out in 96-well plate. Fluorescein (48 nM) was incubated for 15 min at 37 °C in the presence of assayed triterpenes and Trolox. Triterpene concentrations ranged from 12.5 to 400 μM, whereas Trolox concentrations were from 12.5 to 100 μM. The assay was initiated by the addition of AAPH (100 mM) and incubation was carried out at 37 °C during 210 min in a microplate reader (TECAN GENios plus). The fluorescence (λ_{exc} = 485 nm and λ_{em} = 520 nm) readings were taken every 5 min after AAPH addition. Final fluorescence measurements were expressed relative to the initial reading (f_0). Sample fluorescence values were corrected for the blank value. Data were expressed as μM Trolox equivalent (TE). All determinations were carried out in triplicate.

2.4. Copper chelating capacity

Stock solutions of each tested compounds were prepared in ethanol. Final solutions of 50 μM of each compound were prepared in a cuvette containing PBS, and CuSO$_4$ (final concentrations 50 and 100 μM) was added. Samples without copper were run as blanks (Belinky et al., 1998). Absorption spectra were recorded from 190 to 400 nm in a UV–Vis spectrophotometer (Varian Cary 50 Bio). Copper chelating ability was evaluated by absorbance change after subtracting the blank (Rahman et al., 1990).

2.5. In vitro LDL-supported thrombin generation

LDL-supporting thrombin generation was assessed as previously described by Mesa et al. (2004) with some modifications. Briefly, 55.5 ng of LDL was incubated in a final concentration of 1.85 μM prothrombin, 18.5 nM FV and 12.9 nM factor Xa at 37 °C for 15 min, in a 96-well microtitre plate. Afterwards, 5 μL of tested triterpenes (10 and 20 μM in ethanol) were added to the mixture followed by 5 μL CaCl$_2$ (2.5 mM in 0.5% BSA Tris–HCl, pH 7.5) to initiate the formation of thrombin. The plate was immediately incubated at 37 °C for exactly 10 and 20 min. At these time points, 25 μL of 4 mM S-2238 chromogenic thrombin substrate were added. The reaction was stopped after 5 min by adding 150 μL of 1 M citric acid and the absorbance was read at 405 nm against a thrombin standard in an ELISA plate reader (Synergy HT, BIO-TEK Instruments, VT, USA). Controls, containing the same amount of ethanol instead of the ethanolic triterpenes solutions, were also run. Data were expressed as units of thrombin/mL. Measurements were performed in triplicate.

2.6. Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). Before any statistical analysis, all variables were checked for normality and homogeneous variance by using Kolmogorov–Smirnov and Levene tests, respectively. As all variables