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Characterisation of the antioxidant effects of *Aesculus hippocastanum* L. bark extract on the basis of radical scavenging activity, the chemiluminescence of human neutrophil bursts and lipoperoxidation assay

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Abstract. – OBJECTIVES, Oxidative stress is increasingly recognised as a pivotal factor that plays a number of roles in the inflammatory response to environmental signals. It has been claimed that *Aesculus hippocastanum* extracts have antioxidant and anti-inflammatory activity, but these claims are mainly based on the results of chemical reactions and folk-medicine.

MATERIALS AND METHODS, The aim of this study was to examine whether a bark extract of *Aesculus hippocastanum* interferes with reactive oxygen/nitrogen species (ROS/RNS) during the course of human neutrophil respiratory bursts, and to establish the lowest concentration at which it still has antioxidant activity by means of luminol amplified chemiluminescence (LACL). We also studied its ability to counteract lipid peroxidation (LPO) in human cells. Before investigating its antioxidant effects on human cells, we analysed its scavenging activity against $ABTS^{\cdot+}$, hydroxyl radical, superoxide anion, and Fremy's salt (those last three by means of electron paramagnetic resonance (EPR) spectrometry).

RESULTS, The extract of *Aesculus hippocastanum* exerted its anti-ROS/RNS activity in a concentration-dependent manner with significant effects being observed for even very low concentrations: 10 μg/ml without L-Arg, and 5 μg/ml when L-Arg was added to the fMLP test. The LPO assay confirmed these results, which were paralleled by the EPR study.

CONCLUSIONS, These findings are interesting for improving the antioxidant network and restoring redox balance in human cells, and extend the possibility of using plant-derived molecules to antagonise the oxidative stress generated in living organisms when the balance is in favour of free radicals as a result of the depletion of cell antioxidants.

$ABTS^{\cdot+}$ = 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)

Key Words:

Aesculus hippocastanum bark extract, Human neutrophils, Respiratory bursts, Chemiluminescence, Lipoperoxidation, Electron paramagnetic resonance, Antioxidant activity.

Introduction

The catalytic action of a number of enzymes and electron transport processes involve a one-electron transfer that yields free radical intermediates and reactive oxygen species (ROS) in a wide range of biological processees[1]. Moderate concentrations of ROS play an important role as regulatory mediators in signal transduction processes and major cellular events (e.g. the transcriptional activation of nuclear factor kB, normal cell growth, gene expression, cellular proliferation, cellular senescence, and programmed cell death)[2-4]. An excessive amount of ROS may arise from excessive neutrophil burst release or less well regulated sources such as the mitochondrial electron transport chain or the exogenous sources. If these excess is not balanced by endogenous antioxidant defences a oxidative stress can induce pathological situations (e.g. inflammation, mutation, carcinogenesis, aging, etc.)[4,5] that give rise to various diseases[6]. Oxygen is an unusual molecule in that it has two unpaired electrons with parallel spins (biradical). In order to overcome spin restriction, oxygen prefers to accept electrons one at a time, and the sequential addition of electrons leads to the formation of ROS[7]. Because of the ubiquity of molecular oxy-

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gen in aerobic organisms, and the fact that it readily accepts electrons, oxygen-centred free radicals are continuously produced[8].

In order to decrease oxidative stress (for example during over-regulated human polymorphonuclear neutrophil (PMN) respiratory bursts and the associated inflammation) a rational strategy is to remove the starting cause: i.e. the specific toxic effector molecules. However, this is frequently difficult and an alternative is to increase antioxidant defences by administering agents with antioxidant activity[8-10].

This second approach offers more practical therapeutic possibilities and various antioxidant molecules are available. However, only a few have been adopted in clinical practice and, nowadays, there is a growing interest in the biochemical functions of natural vegetable, fruit, and medicinal plant extracts endowed with antioxidant activity, which are being increasingly used in specific antioxidant formulations or alimentary integrators.

It has been claimed that *Aesculus hippocastanum* extracts have antioxidant and anti-inflammatory activity[11,12], mainly on the basis of the results of chemical reactions and folk medicine that are not always completely reliable because of the lack of a control drug and variability. For example, it is still unclear whether they are capable of antagonising the oxidative burden released by human PMNs during respiratory bursts, one of the main sources of oxidant radicals.

The aim of this study was to examine whether a bark extract of *Aesculus hippocastanum* interferes with ROS and RNS during the course of human PMN respiratory bursts, and to establish the lowest concentration at which it still has antioxidant activity by means of luminol amplified chemiluminescence (LACL). We also studied its ability to counteract lipid peroxidation (LPO) in human cells. Before investigating its antioxidant effects on human cells, we analysed its scavenging activity against $ABTS^{\bullet+}$, hydroxyl radical ($HO^{\bullet}$), superoxide anion ($O_2^{\bullet}$), and Fremy's salt (the last three by means of electron paramagnetic resonance (EPR) spectrometry).

Materials and Methods

Plant Material and Extraction

A commercial preparation of a hydro-alcoholic extract of *Aesculus hippocastanum* was obtained from EPO (Estratti Piante Officinali, srl, Milan, Italy), and the identification was done according to the monography "Aesculum hippocastanum ex cortice, ethanol decoctum", S. Benynes, German Homeopathic Pharmacopoeia, RWS Group, London, 2000, sect.1/3A. The raw material used for the extraction consisted in fragments (1-3 cm) of *Aesculus hippocastanum* bark collected in spring and autumn, which was dried and minced, placed in a steel container, and submerged in a solution of water and ethanol (30:70%) at a temperature of 37°C for 24 hours. The mixture was filtered and concentrated at a temperature of 65°C under vacuum at 70 cm Hg, and then dried by means of a spray dryer. On the extract were determined the total phenolic compound levels (Folin-Ciocalteau's reagent)[13], the total flavonoids[14], and proanthocyanidins[15].

Scavenging of $ABTS^{\bullet+}$

The free radical scavenging capacity of various extract concentrations (80 μg/ml to 2.5 μg/ml) was studied using the ABTS radical cation decolorisation assay, which is based on the reduction of $ABTS^{\bullet+}$ radicals by antioxidants of the plant extract tested. We used the method of Re et al[16]. Briefly, ABTS (Sigma Chemical Co., MO, USA) was dissolved in deionised water to a 7 mM concentration, and ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting the ABTS solution (1 mL) with 2.45 mM potassium persulfate (10 μL) (Sigma) and leaving the mixture (stock solution) in the dark at room temperature for 12-16 h to give a dark blue solution. For this study, the $ABTS^{\bullet+}$ solution was diluted in deionised water to an absorbance of 0.700 (±0.020) at 734 nm, and an appropriate solvent blank reading was made (A_B). An aliquot of the test sample (100 μL) was mixed with $ABTS^{\bullet+}$ solution (900 μL) in a 1 ml cuvette, and its adsorbance was recorded for 15 min (A_E). All of the solutions were used on the day of preparation, and all of the determinations were carried out in duplicate. The percentage inhibition of $ABTS^{\bullet+}$ was calculated using the formula:

$$\% \text{ inhibition} = [(A_B - A_E)/A_B] \times 100$$

Electron Paramagnetic Resonance (EPR) Spectrometry

EPR spectrometry was used to investigate the antiradical activity of various compounds. By definition, a free radical is a species containing an unpaired electron and, therefore, paramagnetic. This property forms the basis of the physical de-

tection of free radicals by EPR, whereby the magnetic moment exerted by the unpaired electron is detected[17]. EPR not only allows the direct detection of free radicals, but also detects the activity of molecules with antiradical activity[17].

Fenton Reaction Model System with EPR Detection of Hydroxyl Radical ($HO^•$)

The first series of tests used the spin trapping method[18], which is based on the rapid reaction of many radicals with certain chemical acceptor molecules (spin trapping agents) to produce more stable secondary radicals. The diamagnetic spin trap nitrone DMPO (5,5-dimethyl-1-pyrrolidine-N-oxide) (Sigma) was added to the reaction mixture to produce the relatively long-lived free radical products DMPO-OH, which can be easily investigated by EPR. The activity of the *Aesculus hippocastanum* extract (from 80 μg/ml to 2.5 μg/ml) was evaluated by assessing its ability to scavenge the hydroxyl radical ($HO^•$), the most potent active oxygen species[19]. To obtain the Fenton reaction, the final concentrations were: $FeSO_4 \bullet 7H_2O$ 0.31 mM/L (Sigma), 2Na EDTA 0.34 mM/L (Sigma) and H_2O_2 0.31 mM/L (Sigma) and DMPO 0.78 mM/L (Sigma). The Fenton reaction was initiated by mixing the Fe-EDTA solution with the extract or phosphate buffered saline (PBS) (0.1 mM/L, pH 7.4) (control), and then adding the H_2O_2 solution. The hydroxyl radical generated by a standard Fenton reaction was trapped using DMPO as previously described[20,21] with slight modifications.

The solutions were carefully mixed in a glass tube and then placed in a 100 μl capillary tube for EPR analysis. The EPR spectra were recorded after exactly 1 min. The resulting DMPO-OH (consisting of a quartet of resonances with 1:2:2:1 relative intensities) was detected using an X-band EPR spectrometer (mod. Miniscope MS 200, Magnettech, Berlin, Germany), whose parameters were: field modulation 100 KHz, modulation amplitude 2000 mG, field constant 60 s, centre field 3349.39 G, sweep width 99.70 G, X-band frequency 9.64 GHz, attenuation 7, and gain 100. The percentage $HO^•$ scavenging activity of the assayed solution was expressed by means of the formula: $100 \bullet (h_0 - h_X)/h_0$ [%], where h_x *and* h_o are the relative heights of the highest resonance signal (mm) of the DMPO-OH adduct spectra in a reaction mixture without and with the *Aesculus hippocastanum* extract.

KO_2 in Crown-Ether as a Source of Superoxide Anion ($O_2^{•-}$)

In the second series of tests, the EPR analysis was based on the spin trapping of the superoxide radical ($O_2^{•-}$) generated by potassium superoxide (KO_2) in dimethylsulpxide (DMSO) with the addition of 18-crown-6-ether to complex K^+ because, under these conditions, a DMPO-OOH adduct is observed[21,22]. A typical reaction mixture contained 7.29 mM/L KO_2 (Sigma), 9.013 mM/L crown-ether (Sigma) in DMSO and 14.29 mM/L DMPO (Sigma), and the effects of the same amounts of the extract as in the previous test were investigated.

The reaction mixture was stirred and transferred into a 100 μl capillary tube for EPR analysis, and the EPR spectra were recorded after exactly 30 sec. The resulting DMPO-OOH was detected using an X-band EPR spectrometer (mod. Miniscope MS 200, Magnettech, Berlin, Germany), whose parameters were: field modulation 100 KHz, modulation amplitude 2500 mG, field constant 45 s, centre field 3349.39 G, sweep width 147.76 G, X-band frequency 9.64 GHz, attenuation 7, and gain 800. The intensity of EPR was expressed by means of the formula: $100 \bullet (h_0 - h_X)/h_0$ [%], where h_x *and* h_o are the relative heights of the highest resonance signal (mm) of the DMPO-OOH adduct spectra in a reaction mixture without and with the extract.

EPR Assay Based on the Reduction of Fremy's Salt Radical

A third series of tests were performed using Fremy's salt (potassium nitrosodisulfonate or $[(KSO_3)_2NO]$), a persistent water-soluble radical[22]. A typical reaction mixture contained 2.5 μM/L of Fremy's salt (Sigma), 0.1 M/L phosphate buffer, and the same amounts of the extract as in the previous tests.

The mixture was stirred and transferred into 100 μl glass capillary tube, and the EPR spectra were recorded after 15 min at room temperature using a spectrometer (mod. Miniscope MS 200, Magnettech, Berlin, Germany), operating on the X-band. The typical instrument settings were: field modulation 100 KHz, modulation amplitude 2000 mG, field constant 60 s, centre field 3350.27 G, sweep width 99.70 G, X-band frequency 9.64 GHz, attenuation 7, and gain 100. The intensity of the EPR signal was measured at the height of the first line. The scavenging activity of the extract was defined as $100 \bullet (h_0 - h_X)/h_0$ [%], where h_0 is the height of the first line in the

EPR spectrum of Fremy's free radicals in the blank, and h_X the height of the first line in the EPR spectrum of Fremy's free radicals in the presence of the extract.

Human PMN Harvesting

Peripheral venous blood (5 ml) drawn from healthy adult donors was stratified on 3 ml of a Polymorphprep cell separation medium (Sentinel Ch., Milan, Italy), and the PMNs were separated by means of density gradient centrifugation. After centrifugation, the upper mononuclear cell band was discarded, and the lower PMN band was washed in RPMI 1640 medium containing glutamine (Sigma). When necessary, any residual erythrocytes in the granulocyte preparation were lysed using a 0.15 mol/l NH_4Cl solution (pH 7.4). After the aggregates were disrupted by being passed through a needle with an internal diameter of 150 μm, the PMNs were collected, washed in Hank's buffered salt solution (HBSS), and tested for viability by means of Trypan blue exclusion. The number of cells in the final cell suspension used for each test was adjusted by counting in a Burker chamber by means of a microscope Olympus, mod BH2, Milan, Italy (with interference contrast equipment).

Measurement of Oxidative Burst Responses by Luminol Amplified Chemiluminescence (LACL)

This method has been widely used to detect the PMN production of ROS/RNS under various conditions[23-25]. In order to yield light, luminol has to undergo two-electron oxidation and form an unstable endoperoxide, which decomposes to an excited state (3-aminophthalic acid), and then relaxes to the ground state by emitting photons[26,27] that are amplified by the phototube of a luminometer. PMN oxidative bursts are associated with the generation of superoxide anions, hydrogen peroxide, oxygen radicals, hydroxyl radicals and hypochlorous acid (ROS). As luminol degradation by ROS is associated with luminescence, the inclusion of luminol in the reaction medium provides a sensitive means of detecting PMN respiratory bursts.

LACL was investigated using the soluble stimulants N-formyl-methionyl-leucyl-phenylalanine (fMLP) which is a bacterial tripeptide that is frequently used to stimulate PMN respiratory bursts and acts via a specific receptor.

The measurements were made using a slightly modified version of the procedure described by Briheim and Dahlgren[23].

Briefly, 0.1 ml of a PMN suspension ($1x10^6$ cells/ml) plus 0.2 ml of 2×10^{-5} mol/l of luminol (Sigma) were put into a 3 ml flat-bottomed polystyrene vial. The vial was placed in the light-proof chamber of a luminometer (mod.1250, Bio Orbit, Luminometer, Turku, Finland), and the carousel was rotated to bring the sample in line with the photomultiplier tube in order to record background activity. Subsequently, fMLP $5x10^{-7}$ mol/l was added to reach a final volume of 1 ml, and the resulting light output was continuously recorded in millivolts on a chart recorder and simultaneously by means of a digital printout set to record intervals of 1-10 sec. All of the constituents of the mixture were kept at 37°C during the reaction by passing water from a thermostatically controlled circulation system through a polished hollow metal sample holder. No mixing took place during the recordings. The gain control was set to give a recording of 10 mV for a built-in standard. A background subtraction control zeroed the instrument before the addition of fMLP. The LACL response patterns were identified by calculating peak values (mV) and the times to peak values (min, sec). The effect of the *Aesculus hippocastanum* extract was evaluated at concentrations ranging from 80 μg/ml to 2.5 μg/ml. The incubation time was 15 min at 37°C.

A second series of human PMN tests was performed in the same way, but L-Arg (170 μg/ml) (Sigma) was added to the medium incubating the PMNs as a NO donor in order to be able to read the NO-derived peroxynitrite radical by means of LACL.

In these tests, the effects of the extract were evaluated at concentrations ranging from 80 μg/ml to 2.5 μg/ml. The incubation time was 15 min at 37°C.

Lipid Peroxidation (LPO) Measurement

The A549 human lung carcinoma epithelial-like cell line was obtained from the ATCCC (American Type Cell Culture Collection). The cells were cultured in RPMI-1640 medium supplemented with 1% penicillin-streptomycin, 0.8% pyruvic acid, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) in 100 mm plastic tissue culture dishes, and maintained in a humidified atmosphere at 37°C and 5% CO_2.

Exposure was started at 80% confluence. Antioxidant treatment was done prior to oxidant exposure. The cells were first treated with different concentrations of the extract for 10 min at 37°C,

5% CO_2, and then hydrogen peroxide solution (4 mM, also used as positive control) was added for two hours, under the same cell growth conditions. Lipid peroxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay, which mainly detects malondialdehyde (MDA), an end-product of the peroxidation of polyunsatured fatty acids and related esters. TBARS was measured by using a slightly modified version of the method of Ahamed et al[28]. The cells were grown in 100 mm tissue culture dishes to approximately 80% confluency, and after treatment, the medium was removed and the cells were washed with PBS. The cell suspension was lysed in liquid nitrogen and then homogenised in ice-cold PBS. One millilitre of TBARS solution (0.375% TBA, 20% TCA, 25% HCl 1N) was added to the samples, which were incubated for 30 min to 70°C, cooled in ice and then centrifuged at 10,000 g for 5 min. The absorbance of the supernatants (i.e. the absorbance of TBARS) was measured spectrophotometrically at 532 nm. The concentration of TBARS was determined using a standard curve of MDA obtained from the acid hydrolysis of tetraethoxypropane (TEP). The results were normalised against protein levels in each sample, as measured using the method of Lowry et al[29], and expressed as nmol of MDA/mg of cell protein.

Statistical Analysis

Four assays of each concentration were made in each test, and the statistical significance of the differences was calculated by means of one-way ANOVA followed by multiple paired comparisons using Dunnett's test. The differences were considered statistically significant when the p value was ≤ 0.05.

Results

The effects of the bark extract of *Aesculus hippocastanum* on the reduction in $ABTS^{\bullet+}$ radicals are shown in Figure 1; the lowest concentration that was still active was 5 μg/ml. From this concentration to 80 μg/ml (the highest concentration investigated), there was a significant concentration-dependent inhibition of $ABTS^{\bullet+}$ radicals (from 96.54 ± 1.27% to 16.91 ± 0.64%)

The EPR adduct DMPO-OH was significantly reduced by the bark extract at concentrations ranging from 80 μg/ml to 20 μg/ml (from 48.66 ± 2.74% to 21.98 ± 1.25%) (Figure 2).

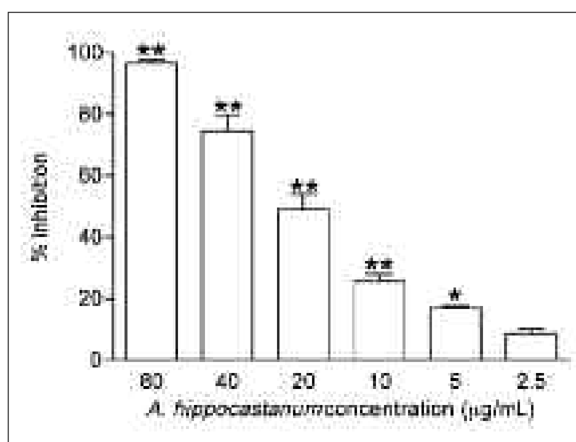

Figure 1. Effects of various concentrations of *Aesculus hippocastanum* extract on ABTS radical cation ($ABTS^{\bullet+}$) (** = $p \leq 0.01$; * = $p \leq 0.05$).

Figure 3 shows representative spectra of the adduct DMPO-OOH (from KO_2+crown-ether). There was a significant reduction at bark extract concentrations ranging from 20 μg/ml to 80 μg/ml (from 25.58 ± 3.68% to 56.31 ± 2.60%) (Figure 3).

The isotropic spectrum of Fremy's salt consists of three single narrow peaks that arise from the interaction of the unpaired electron spin with the nuclear spin of nitrogen. This triple resonance was significantly reduced at bark extract concentrations ranging from 80 μg/ml to 10 μg/ml (from 93.00 ± 1.22% to 22.19 ± 3.48%) (Figure 4).

From a general point of view, all of these findings showed the presence of scavenging activity, with the relative intensity varying depending on the type of radical.

Microscopic examination of the neutrophil suspensions showed that the population of neutrophils was always ≥95%, and the viability determined by means of trypan blue dye exclusion was always ≥94%. None of the concentrations of the *Aesculus hippocastanum* extract used in the fMLP tests affected PMN viability. Figure 5 shows the effects of the various *Aesculus hippocastanum* extract concentrations on the LACL of fMLP-induced PMN respiratory bursts. The lowest concentration that still had significant antioxidant activity was 10 µg/ml. From this concentration to 80 µg/ml (the highest concentration investigated), there was a significant concentration-dependent inhibition of peak chemiluminescence. The times to peak chemiluminescence were generally similar at the various concentrations, and were not significantly different from those of the controls.

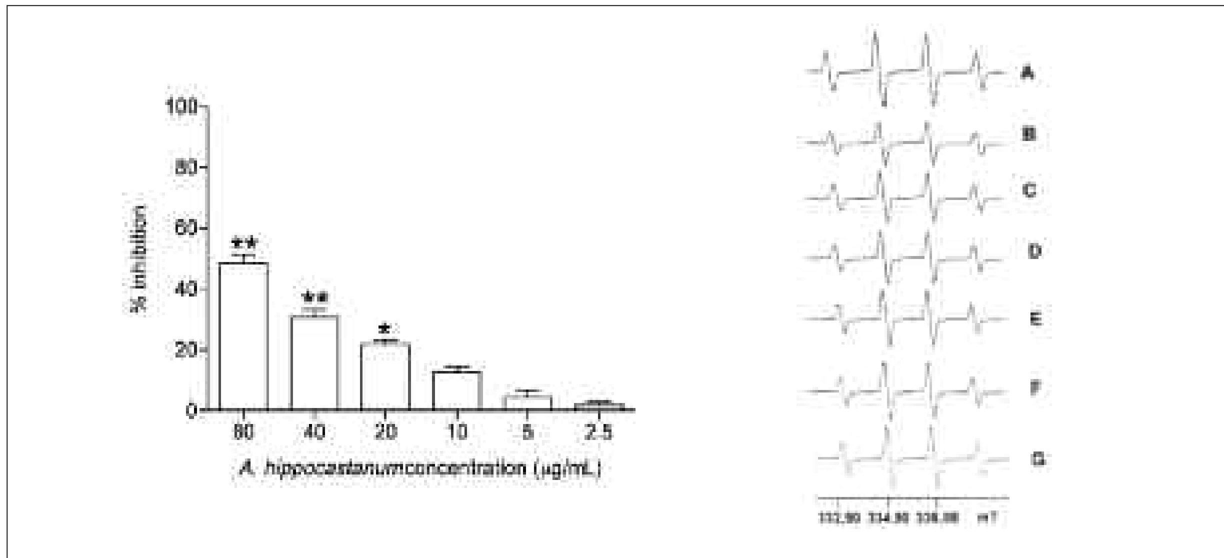

Figure 2. Effects of various amounts of *Aesculus hippocastanum* extract on the HO$^{\bullet}$ radical. Panel A: Percentage quenching effect of various amounts of the extract using DMPO to trap the HO$^{\bullet}$ radical (** = $p \leq 0.01$; * = $p \leq 0.05$). Panel B: Examples of EPR spectra: A = control; B-G = effects of concentrations ranging from 80 μg/mL to 2.5 μg/mL.

When L-Arg was added to the reaction medium as a NO donor, baseline LACL increased approximately 3-4 times. The inhibiting behaviour of the same concentrations of *Aesculus hippocastanum* under these new conditions was confirmed, and parallelled those previously obtained with fMLP without the addition of L-Arg to the medium. The lowest concentration that significantly reduced LACL was 5 µg/ml; from that to 80 µg/ml, the concentration-dependent inhibition of peak chemiluminescence was significant (Figure 6). The time to peak LACL was not significantly different from that of the controls.

The findings of lipoperoxidation (LPO) test are shown in Figure 7. Exposure of A549 cells to H_2O_2 (4 mM for two hours) increased lipid per-

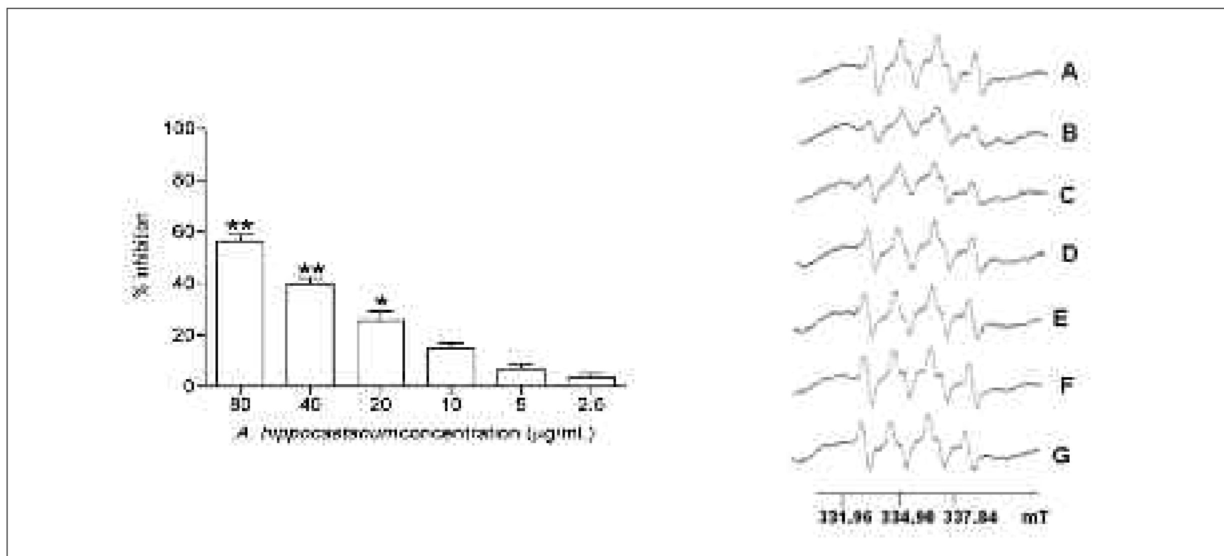

Figure 3. Effects of various amounts of *Aesculus hippocastanum* extract on the $O_2^{\bar{\bullet}}$ radical. Panel *A*: Percentage quenching effect of various amounts of the extract using DMPO to trap the $O_2^{\bar{\bullet}}$ radical (** = $p \leq 0.01$; * = $p \leq 0.05$). Panel *B*: Examples of EPR spectra: A = control; B-G = effects of concentrations ranging from 80 μg/mL to 2.5 μg/mL.

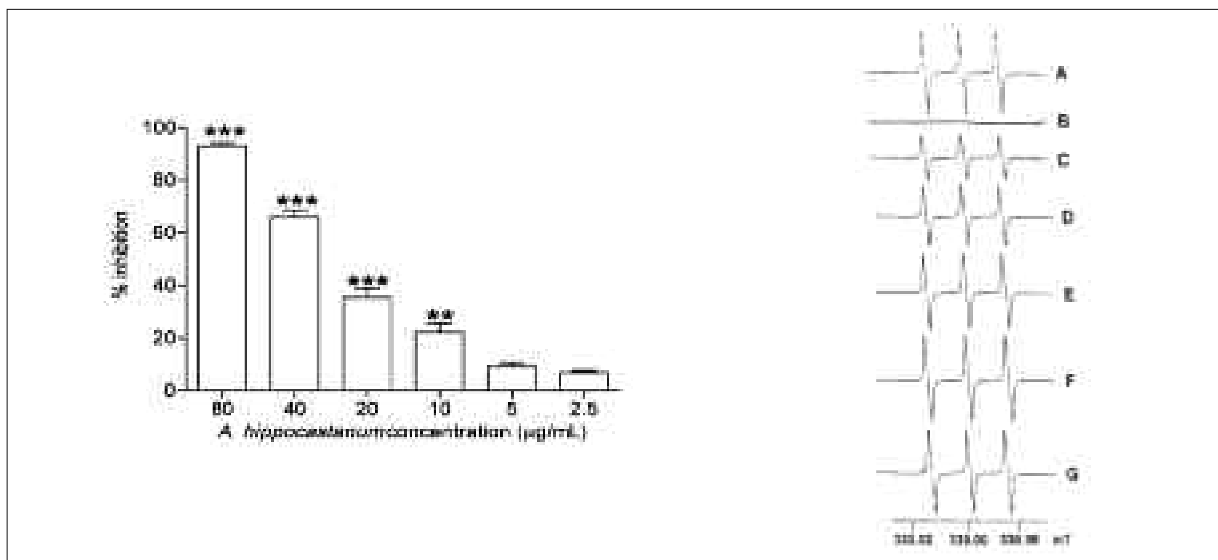

Figure 4. Effects of various amounts of *Aesculus hippocastanum* extract on Fremy's salt radical (*** = $p \leq 0.001$; ** = $p \leq 0.01$). Panel *A*: Percentage quenching effect of various amounts of the extract to trap the Fremy's salt radical. Panel *B*: Examples of EPR spectra: A = control; B-G = effects of concentrations ranging from 80 µg/mL to 2.5 µg/mL.

oxidation membranes at TBARS levels of 214% in comparison with control value. Pretreatment with 80 and 40 µg/ml extract significantly inhibited the induced damage.

Discussion

Despite the presence of a cellular antioxidant defence system to counteract the oxidative damage caused by ROS, oxidative damage accumulates during the life cycle because of radical-related damage to DNA, proteins and lipids. The role of free radicals is, therefore, gaining increasing attention as so many pathological phenomena are related to changes in cell redox status. The oxidative stress-activated signalling cascade upregulates the genes controlling inflammatory processes[5], and so reducing oxidative stress by controlling ROS or RNS levels using plant extracts with antioxidant activity, means interferring with the signal transduction pathways of inflammation.

It has been experimentally documented that *Aesculus hippocastanum* extract has protective

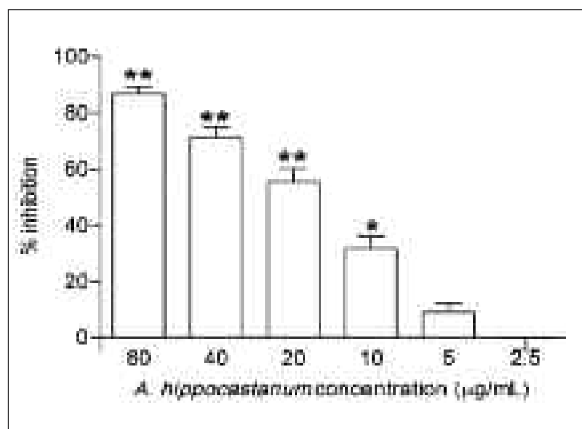

Figure 5. Effects of various concentrations of *Aesculus hippocastanum* extract on the LACL of PMN respiratory bursts induced by fMLP (** = $p \leq 0.01$; * = $p \leq 0.05$).

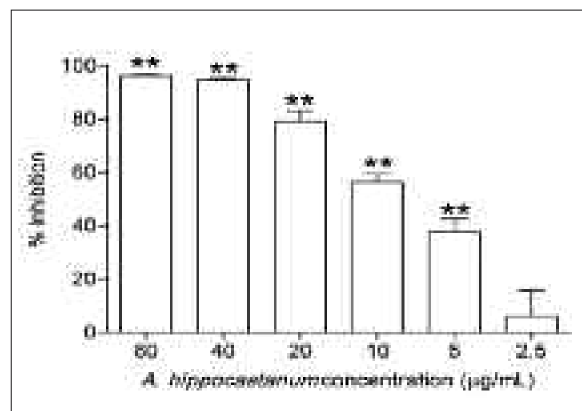

Figure 6. Effects of various concentrations of *Aesculus hippocastanum* extract on the LACL of PMN respiratory bursts induced by fMLP after L-Arg incubation (** = $p \leq 0.01$).

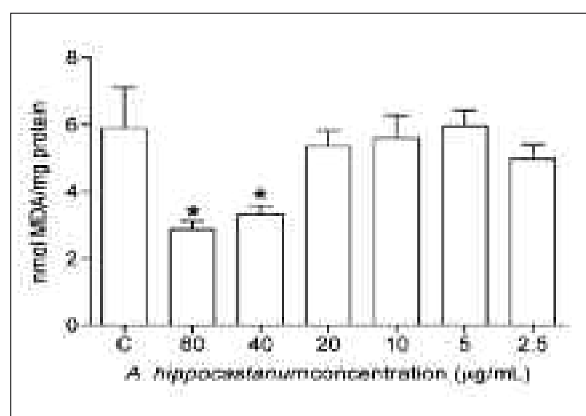

Figure 7. Effects of various concentrations of *Aesculus hippocastanum* extract on LPO assay in A549 cells. Results are expressed in nmol MDA/mg proteins. C = H_2O_2 damage (* = $p \leq 0.05$).

veinotonic, antiedemigenic, and anti-inflammatory activity[30-35], particularly on the microcirculation. Clinically, extracts have been used to treat venous stasis and venous insufficiency[36-40]. Our findings show that *Aesculus hippocastanus* bark extract has interesting concentration-dependent antioxidant activity, confirmed by EPR, and the scavenging of $ABTS^{\bullet +}$. Our findings are in line with the activity described by other Authors[30,40], but extend our knowledge (never previously investigated) of the smallest scavenging concentration still capable of counteracting the hydroxyl radical ($HO^{\bullet}$), the superoxide anion ($O_2^{\bullet}$), Fremy's salt, and $ABTS^{\bullet +}$ radical: respectively 20, 20, 10 and 5 μg/ml. In addition, our LACL findings concerning the effects of *Aesculus hippocastanum* extract on human PMNs (never previously investigated), whose respiratory bursts release a considerable burden of ROS and RNS, show that they are still present in human cells at concentration-dependent manner (confirmed also by LPO data). They further extend our knowledge by indicating that the extract exerts its anti-ROS and anti-RNS (peroxynitrite) activity in a concentration-dependent manner and has significant effects at even very low concentrations: 10 μg/ml for the test without L-Arg and respectively 5 μg/ml when L-Arg was added to the fMLP tests.

As the aim of the study was to characterize the antioxidant activity of *Aesculus hippocastanum* bark extract (mainly on human PMNs, one of the main sources of oxidant radicals), we did not investigate the effects of the individual molecules present in the extract. However, the anti-oxidant and the anti-inflammatory properties (which are interconnected) of *Aesculus hippocastanus* bark extract are mainly attributable to their secondary metabolites content: total phenolic compound level 381.3 mg/g (eq.rutine), total flavonoids 150.8 mg/g, total amount escine 122.5 mg/g, and proanthocyanidins 11.5 mg/g.

Conclusions

Our findings are interesting for improving the antioxidant network and restoring redox balance to human cells, and extend our possibility of using plant-derived molecules to antagonise the oxidative stress generated in living organisms when the balance is in favour of free radicals as a result of the depletion of cell antioxidants.

The anti-oxidant and anti-inflammatory effects of *Aesculus hippocastanum* bark extract seem to be clear, but its molecular mechanisms have not yet been fully investigated. Its effects on the arachidonic acid cascade, serine proteases, protein kinase C and the nuclear factor-kB (NFkB) signalling pathway should be further elucidated in order to complete the anti-oxidant and anti-inflammatory profile.

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