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Full Length Research Paper

Antioxidant activity of *Melilotus officinalis* extract investigated by means of the radical scavenging activity, the chemiluminescence of human neutrophil bursts and lipoperoxidation assay

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Plants have been useful sources of new biologically active compounds. However, traditional ethno-medical approaches may draw attention to one or other clinical claim, but only specific scientific investigation can validate their effects. The aim of this study was to examine whether an extract of *Melilotus officinalis* leaves 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 $ABTS^{\bullet+}$, hydroxyl radical, superoxide anion, and Fremy's salt (the last three by means of electron paramagnetic resonance [EPR] spectrometry). The extract of *M. officinalis* exerted its anti-ROS/RNS activity in a concentration-dependent manner, with significant effects being observed for even very low concentration: 20 µg/ml without L-arginine (L-Arg) and 10 µg/ml when L-Arg was added to the formyl-methionyl-leucyl-phenylalanine (fMLP) test. LPO assay confirmed these results, which were paralleled by the EPR study. 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.

Key words: *Melilotus officinalis* extract, antioxidant activity, human neutrophils, respiratory bursts, chemiluminescence, lipoperoxidation, electron paramagnetic resonance.

INTRODUCTION

Majority of the living organisms exert their vital functions in an environment in which oxygen is the principal component, and they use it as an acceptor of electrons to produce energy for their metabolic pathways. During these reactions, free radicals (particularly reactive oxygen/nitrogen species [ROS/RNS] and their intermediates) are continuously generated and under these conditions, they must be considered useful and normal physiological reactions.

This is because the generation of ROS/RNS and free radicals is "controlled": that is, their production follows well-defined pathways that are useful for the functioning of cells, subcellular organules, and enzymes, as well as the synthesis and metabolism of biomolecules (Finkel, 2003; Rahaman et al., 1998; Schoonbroodt et al., 2000). However, an excessive amount of radicals may arise from various sources including excessive neutrophil burst release or less well-regulated sources such as the mitochondrial electron-transport chain or exogenous sources, and unless balanced by endogenous antioxidant defenses, these can lead to oxidative stress. The ubiquitous nature of the targets of these radicals (proteins, lipids, carbohydrates, DNA) explains the wide

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spectrum of damage they cause in living organisms, which leads to the progressive functional deterioration of cells, tissues and organs, thus inducing pathological situations (e.g. inflammation, mutation, carcinogenesis, aging, degenerative and other diseases) (Schoonbroodt et al., 20003; Braga, 2006a) that give rise to diseases (Freeman et al., 1982; Hoidal, 2001).

A rational strategy to decrease oxidative stress during over-regulated human polymorphonuclear neutrophil (PMN) respiratory bursts and the associated inflammation would be to remove the starting cause, that is, the specific toxic effector molecules. However, as this is frequently complicated and difficult, another possibility is to increase antioxidant defenses by administering agents with antioxidant activity in order to strengthen natural mechanisms (Crystal, 1991; Simon et al., 1985; Braga, 2006b). This second approach offers more practical therapeutic possibilities and various antioxidant molecules are available, although, only a few have been adopted in clinical practice and in this regards, nowadays there is an increasing interest in the biochemical functions of natural extracts from vegetables, fruit and medicinal plants as a useful source of new biologically active compounds, and those endowed with antioxidant activity are being increasingly used in specific antioxidant formulations or alimentary integrators.

Numerous folk-medical data have been reported about the use of *Melilotus officinalis*, but unfortunately they are not completely reliable due to their variability and the absence of a control drug. Furthermore, scientific information concerning antioxidant properties of the extract of *M. officinalis* is still rather scarce. Assessing these properties therefore remains interesting and useful, particularly as a means of finding new sources of natural antioxidants. So, the aim of this study was to examine whether an extract of *M. officinalis* leaves 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 and extraction

A commercial preparation of a *M. officinalis* leaf extract was obtained from Natura Spa (Milan, Italy), and the procedure to get it was as follow: between April and June, manually selected leaves were gathered from plants grown in a certified culture, dried and minced, and placed in a steel container submerged in a solution of water and ethanol (85:15) at a temperature of 70°C for 24 h. 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. The main secondary metabolites were 134.5 mg/g of total phenolic compound (Folin-Ciocalteau's reagent) (Waterman et al., 1994), 48.5 mg/g of total flavonoids (Zou et al., 2004), and 246.3 mg/g of coumarin (Goodwin et al., 1954).

Scavenging of $ABTS^{\bullet+}$

The free radical scavenging capacity of various extract concentrations (160 to 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. The method of Re et al. (1999) was used for the scavenging of ABTS. 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 to 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 absorbance was recorded for 20 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:

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

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 detection of free radicals by EPR, whereby the magnetic moment exerted by the unpaired electron is detected (Antolovich et al., 2002). EPR not only allows the direct detection of free radicals, but also detects the activity of molecules with antiradical activity (Antolovich et al., 2002).

Fenton reaction model system with EPR detection of hydroxyl radical ($HO^{\bullet}$)

The first series of tests used the spin trapping method (Kopani et al., 2006), 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 5.5-dimethyl-1-pyrrolidine-N-oxide (DMPO) (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 *M. officinalis* extract (from 160 to 5 µg/ml) was evaluated by assessing its ability to scavenge the hydroxyl radical ($HO^{\bullet}$), the most potent active oxygen species (Falchi et al., 2006). The final concentrations used to obtain the Fenton reaction were: $FeSO_4 \bullet 7H_2O$ 0.31 mM/L (Sigma), 2Na ethylenediaminetetraacetic acid (EDTA) 0.34 mM/L (Sigma), H_2O_2 0.31 mM/L (Sigma) and DMPO of 0.78 mM/L (Sigma). The Fenton reaction was initiated by mixing the Fe-EDTA solution with the extract or 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 (Valvanidis et al., 2004; Reszkaet al., 1991) 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 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 of $HO^{\bullet}$ scavenging activity of the assayed solution was expressed by means of the formula:

$100 \times (h_0 - h_X) / h_0$ [%],

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 *M. officinalis* extract.

KO_2 in crown-ether as a source of superoxide anion ($O_2^{\bullet}$)

In the second series of tests, the EPR analysis was based on the spin trapping of the superoxide radical ($O_2^{\bullet}$) generated by potassium superoxide (KO_2) in DMSO with the addition of 18-crown-6-ether to complex K^+, because these conditions give rise to a DMPO-OOH adduct (Valvanidis et al., 2004; Reszka et al., 1991). 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 s. The resulting DMPO-OOH was detected using an X-band EPR spectrometer Miniscope MS 200 (Magnettech, Berlin Germany), whose parameters were: 100 KHz field modulation, 2500 mg modulation amplitude, 45 s field constant, 3349.39 G centre field, 147.76 G sweep width, 9.64 GHz X-band frequency, 7 attenuation, and gain 800. The intensity of EPR was expressed by means of the formula:

$100 \times (h_0 - h_X) / h_0$ [%],

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 (Lo Scalzo et al., 2007). 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 a 100 µl glass capillary tube, and the EPR spectra were recorded after 15 min at room temperature using a Miniscope MS 200 EPR spectrometer (Magnettech, Berlin, Germany) operating on the X-band. The typical instrument settings were: 100 KHz field modulation, 2000 mG modulation amplitude, 60 s field constant, 3350.27 G centre field, 99.70 G sweep width, 9.64 GHz X-band frequency, 7 attenuation, 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:

$100 \times (h_0 - h_X) / h_0$ [%],

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, Milano, 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 balance 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 (interference contrast microscopy).

Measurement of oxidative burst responses by LACL

LACL was investigated using the soluble stimulants N-fMLP, 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 et al. (1984). Briefly, 0.1 ml of a PMN suspension (1×10^6 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 1250 (Bio Orbit, 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 5×10^{-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 to 10 s. 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, s). The effect of the *M. officinalis* extract was evaluated at concentrations ranging from 160 to 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 nitric oxide (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 160 to 5 µg/ml. The incubation time was 15 min at 37°C.

LPO measurement

The A549 human lung carcinoma epithelial-like cell line was obtained from the 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 in 100 mm plastic tissue

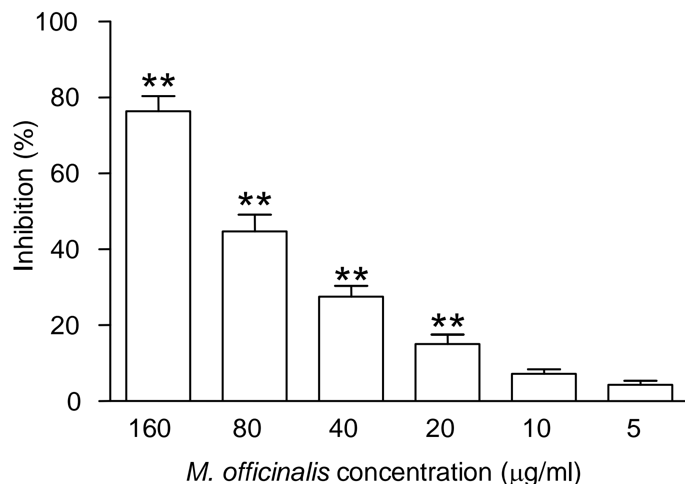

Figure 1. Effects of various concentrations of *M. officinalis* extract on ABTS radical cation ($ABTS^{\bullet+}$) (**p≤0.01).

culture dishes, and maintained in a humidified atmosphere at 37°C and 5% CO_2.

Exposure was started at 80% confluence. Antioxidant treatment was administered before oxidant exposure. The cells were first treated with different concentrations of the extract for 10 min at 37°C, 5% CO_2 and then a hydrogen peroxide solution (4 mM, also used as positive control) was added for 2 h under the same cell growth conditions.

LPO was assessed using the thiobarbituric acid reactive substances (TBARS) assay, which mainly detects malondialdehyde (MDA), an end-product of the peroxidation of polyunsaturated fatty acids and related esters. TBARS was measured using a slightly modified version of the method of Ahamed et al. (2010). The cells were grown in 100 mm tissue culture dishes to approximately 80% confluence 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 1 N) was added to the samples, which were incubated for 30 min at 70° C, cooled in ice and then centrifuged at 10.000 g for 5 min. The absorbance of the supernatants (that is, 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. (1951), and expressed as nmol of MDA/mg of cell protein.

Statistical analysis

Four assays of each concentration were made in each test, and the concentration-effect relationship was expressed in the form of regression line. The statistical significance of the differences was calculated by means of one-way analysis of variance (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 extract of *M. officinalis* leaves on the reduction in $ABTS^{\bullet+}$ radicals are shown in Figure 1; the lowest concentration that was still active was 20 μg/ml. From this concentration to 160 μg/ml (the highest concentration investigated), there was a significant concentration-dependent inhibition of $ABTS^{\bullet+}$ radicals (from 76.39±4.01 to 15.01±2.55%). The EPR adduct DMPO-OH was significantly reduced by the extract at concentrations ranging from 160 to 10 μg/ml (from 82.17±1.31 to 15.47±2.45%) (Figure 2). Figure 3 shows the representative spectra of the adduct DMPO-OOH (from KO_2+crown-ether). There was a significant reduction at extract concentrations ranging from 10 to 160 μg/ml (from 62.63±3.11 to 14.66±2.31%) (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 extract concentrations ranging from 160 to 40 μg/ml (from 63.03±3.77 to 30.29±3.35%) (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.

The method of LACL has been widely used to detect the PMN production of ROS/RNS under various conditions (Briheim et al., 1984; Robinson et al., 1984; Radi et al., 1993). In order to yield light, luminol has to undergone two-electron oxidation and form an unstable endoperoxide that decomposes to an excited state (3-aminophthalic acid), and then relaxes to the ground state by emitting photons (Allen, 1986; Merenyi et al., 1990) 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.

Microscopic examination of the neutrophil suspensions showed that the population of neutrophils was always ≥92%, and the viability determined by means of Trypan blue dye exclusion was always ≥93%. None of the concentrations of the *M. officinalis* extract used in the fMLP tests affected PMN viability. Figure 5 shows the effects of the various *M. officinalis* extract concentrations on the LACL of fMLP-induced PMN respiratory bursts. The lowest concentration that still had significant antioxidant activity was 20 μg/ml. From this concentration to 160 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. When L-Arg was added to the reaction medium as a NO donor, baseline LACL increased approximately 3 to 4 times. The inhibiting behaviour of the same concentrations of *M. officinalis*

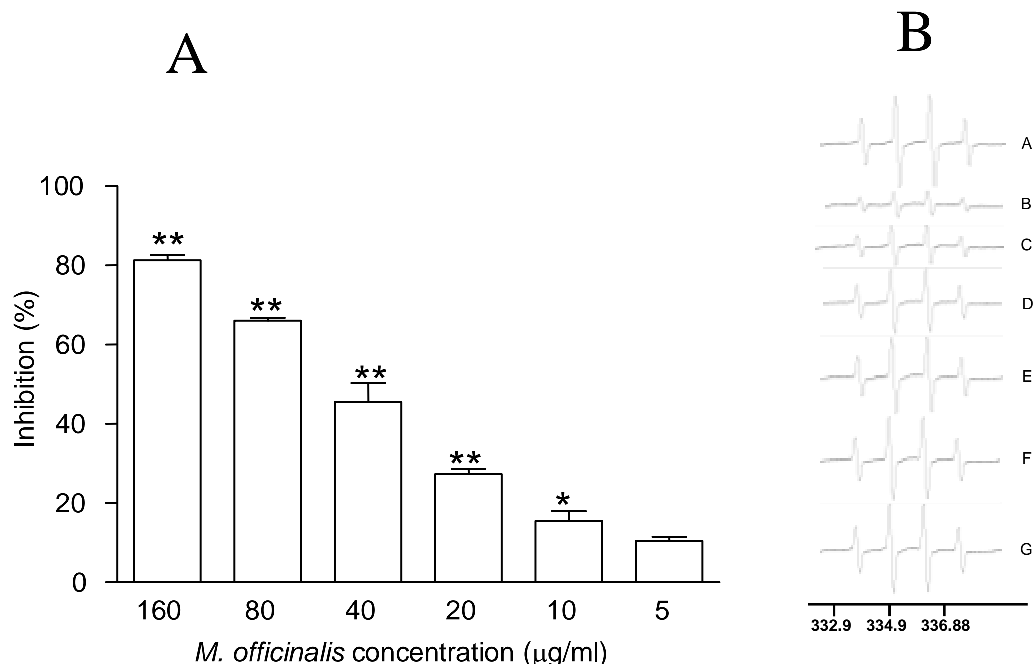

Figure 2. Effects of various amounts of *M. officinalis* extract on the HO• radical. Panel A: Percentage quenching effect of various amounts of the extract using DMPO to trap the HO• radical (**p≤0.01; *p≤0.05). Panel B: Examples of EPR spectra: A=control; B-G= effects of concentrations ranging from 160 to 5 µg/ml.

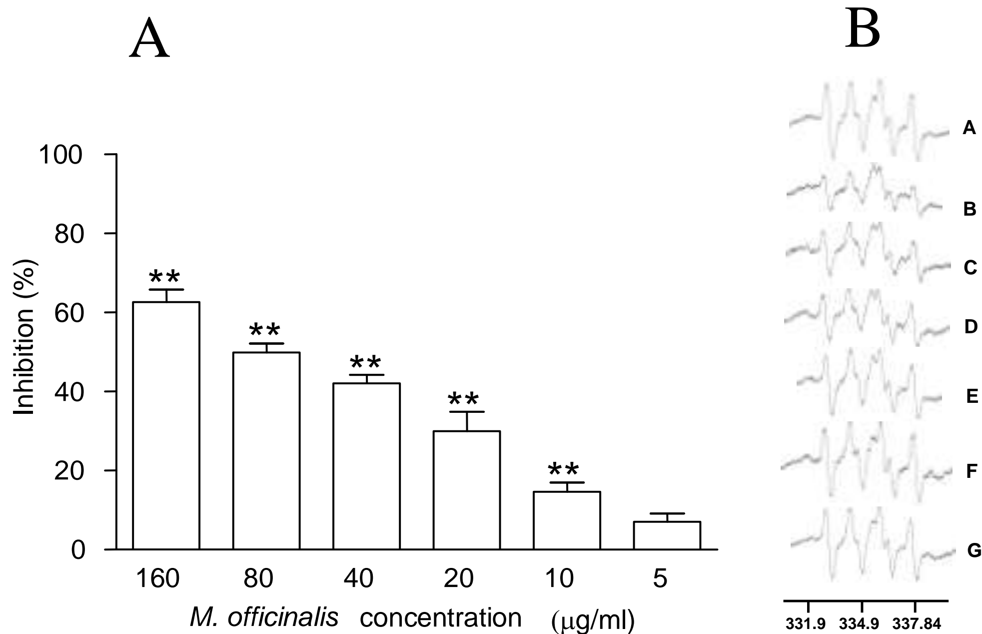

Figure 3. Effects of various amounts of *M. officinalis* extract on the $O_2^{-\bullet}$ radical. Panel A: Percentage quenching effect of various amounts of the extract using DMPO to trap the $O_2^{-\bullet}$ radical (** = p ≤ 0.01). Panel B: Examples of EPR spectra: A=control; B-G= effects of concentrations ranging from 160 to 5 µg/ml.

under these new conditions was confirmed, and paralleled those previously obtained with fMLP without the addition of L-Arg to the medium. The lowest concentration that significantly reduced LACL was 10

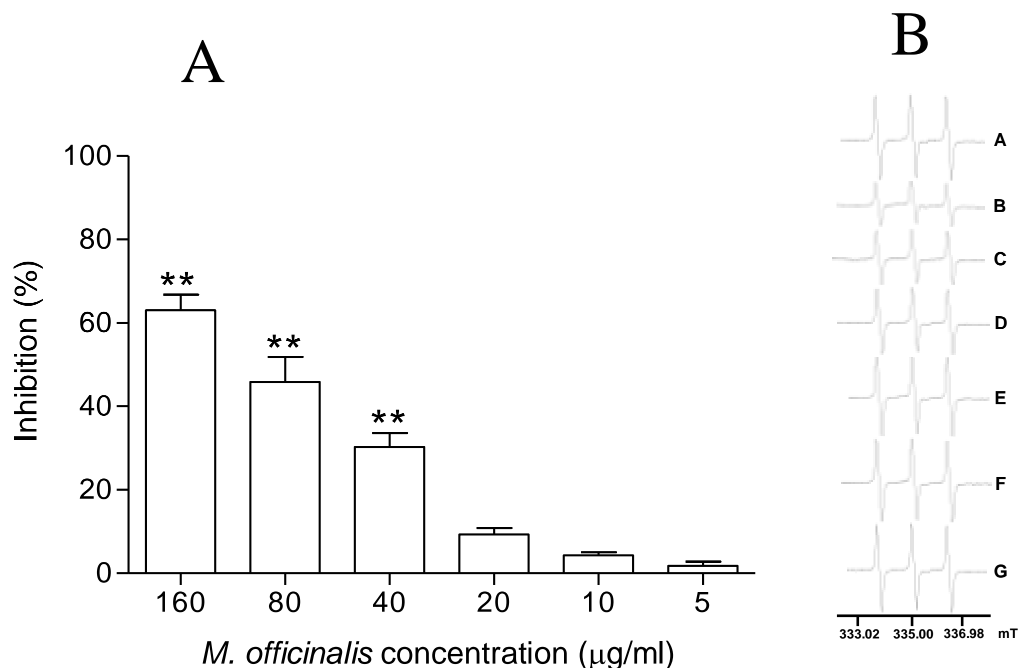

Figure 4. Effects of various amounts of *M. officinalis* extract on Fremy's salt radical (**$p \leq 0.01$). Panel A: Percentage quenching effect of various amounts of the extract to trap the Fremy's salt radical. Panel B: Examples of EPR spectra: A=control; B-G= effects of concentrations ranging from 160 to 2.5 µg/ml.

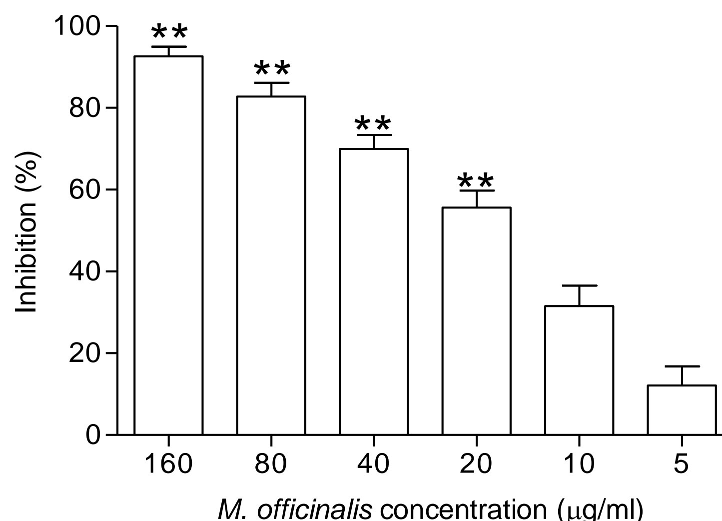

Figure 5. Effects of various concentrations of *M. officinalis* extract on the LACL of PMN respiratory bursts induced by fMLP (**$p \leq 0.01$).

µg/ml; from this concentration to 160 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 the LPO test are shown in Figure 7. Exposure of A549 cells to H_2O_2 (4 mM for 3 h) increased LPO membranes to TBARS levels of 214% in comparison with the basal value. Pretreatment with 160 to 20 µg/ml extract significantly inhibited the induced damage.

DISCUSSION

Many regulatory events depend on the magnitude and changes in ROS/RNS concentrations and the persistent

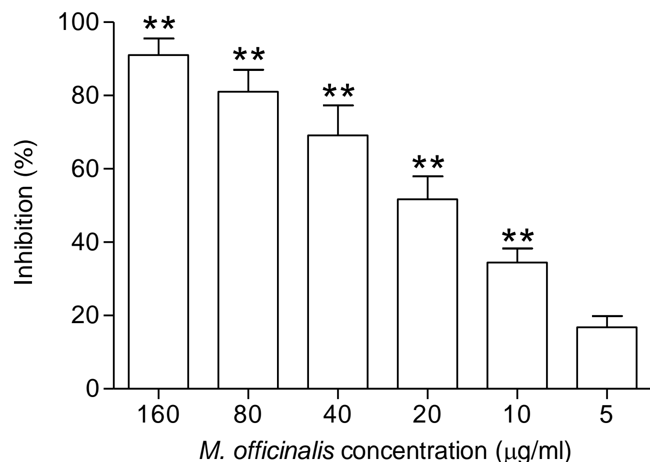

Figure 6. Effects of various concentrations of *M. officinalis* extract on the LACL of PMN respiratory bursts induced by fMLP after L-Arg incubation (**p≤0.01).

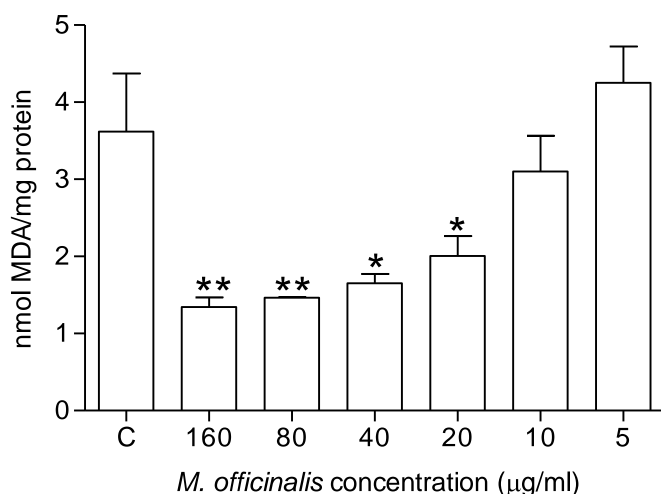

Figure 7. Effects of various concentrations of *M. officinalis* extract on LPO assay in A549 cells. Results are expressed in nmol MDA/mg proteins. C = H_2O_2 damage (**p≤0.01; *p≤0.05).

production of abnormally large amounts of ROS/RNS may dysregulate in signal transduction and gene expression which, in turn, may give rise to pathological conditions (Droge, 2002; Adler et al., 1999).

There has recently been an upsurge of interest in the therapeutic potential of a plant, fruits, vegetables and spices, because their content of antioxidant molecules can be used to antagonise excessive ROS/RNS production. *Melilotus* preparations have long been prescribed in traditional medicine for the treatment of inflammation-related diseases (Pesca-manca et al., 2002), but only a few experimental reports are in literature on its antioxidant and anti-inflammatory action (Pesca-Manca et al., 2002; Pourmourad et al., 2006), and no one testing the activity on human cells.

Our findings show that an *M. officinalis* extract possesses interesting concentration-dependent antioxidant activity that was confirmed by EPR, and the scavenging of $ABTS^{\bullet+}$. Our findings are in line with the presence of an antioxidant activity described by other authors (Hasan Agha et al., 2007; Fiorentino et al., 2007; Milianskas et al., 2004), but extend our knowledge of the smallest scavenging concentration needed to counteract the hydroxyl radical ($HO^{\bullet}$), the superoxide anion ($O_2^{\bullet}$), Fremy's salt and the $ABTS^{\bullet+}$ radical which were respectively 10, 10, 40, and 20 μg/ml. In addition, our LACL data relating to the effects of *M. officinalis* extract on human PMNs (never previously investigated), whose respiratory bursts release major oxidant ROS and RN burden, show that the effects are still present in human cells in a concentration-dependent manner (also confirmed by the 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: 20 μg/ml for the test without L-Arg and 10 μg/ml when L-Arg was added to the fMLP tests, respectively.

Our attention was primarily drawn to the coumarin, because the amount in the extract of *M. officinalis* (246.3 mg/g) was 1.8 times that of total phenolics (134.5 mg/g), and five times that of total flavonoids (48.5 mg/g). We speculated that most of the anti-oxidant activity of the extract could be attributed to coumarin, but this is not completely correct, because as phenolics and flavonoids are also anti-oxidants and possibly influence each other, it is better to consider the concept of the "phytocomplex" of all of the components.

Taken together, these present findings show that the phytocomplex obtained by means of water/ethanol extraction from *M. officinalis* leaves can be considered an interesting anti-oxidant agent as a whole.

Conclusion

Our findings are interesting for the strategy of improving the antioxidant network and restoring redox balance to human cells, and extending 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.

The anti-oxidant and anti-inflammatory effects of *M. officinalis* extract seem to be clear, but its molecular anti-inflammatory mechanisms have not yet been fully investigated.

The effects on the arachidonic acid cascade, serine proteases, protein kinase C and the NFkB signalling pathway should be further elucidated in order to complete the anti-oxidant and anti-inflammatory profile of the extract.

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