European Review for Medical and Pharmacological Sciences 2013; 17: 1987-1999

Comparative antioxidant activity of cultivated and wild *Vaccinium* species investigated by EPR, human neutrophil burst and COMET assay

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Abstract. – OBJECTIVES: The *Vaccinium* (V.) spp. berries are considered a source of antioxidants, mainly belonging to polyphenols, specifically flavonoids and anthocyanins. Wild genotypes generally contain more antioxidants than cultivated counterparts. So, seven different antioxidants assays on extracts from cultivated and wild *Vaccinium* berries were performed, to evaluate their difference in terms of bioactivity on oxidative protection and minimum dosage to have a significant action.

MATERIALS AND METHODS: Four cell-free antioxidant assays (ABTS radical scavenging and electronic paramagnetic resonance using Fremy's salt, superoxide anion and hydroxyl radical), and three assays on human cells (two luminol amplified chemiluminescence, LACL, one on DNA damage, COMET) were used to measure the effects of cultivated blueberry (*V. corymbosum*) and wild bilberry (*V. myrtillus*) on the differently induced oxidative stress. Concentrations vs activity patterns were obtained by successive dilutions of extracts in order to identify both EC50 and minimum significant activity (MSA).

RESULTS: All the assays (except for the hydroxyl radical scavenging) showed a good relationship mainly with anthocyanin and polyphenol content and the significant greater activity of wild *Vaccinium* extracts. In fact, LACL data gave an EC50 of 11.8 and an MSA of 5.2 g were calculated as fresh weight dosage in cultivated berries, compared with lower doses in wild berries, EC50 of 5.7 g and MSA of 3.4 g.

CONCLUSIONS: Wild *Vaccinium* extracts averaged 3.04 and 2.40 fold more activity than cultivated extracts by EC50 and MSA, respectively. COMET assay confirmed the stronger action on DNA protection in wild samples.

Key Words:
Antioxidant assays, Bilberry, Blueberry, Comet assay, Electron paramagnetic resonance, Human neutrophils oxidative burst.

Abbreviations

ABTS = 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid);
LACL = luminol amplified chemiluminescence
COR = cultivated blueberry (*V. corymbosum*)
MYRT = wild bilberry (*V. myrtillus*)
EC50 = extinction capacity of 50%
EPR = electron paramagnetic resonance.

Introduction

Berries of the genus *Vaccinium (V.)* are known to have a significant content of phytonutrients, whose antioxidant action inhibits the free radicals produced under oxidative stress (reactive oxygen species or ROS)[1,2]. Published data comparing the antioxidant action of different fruits and fruit products always rank *Vaccinium* highly[3,4]. From a chemical point of view, the main compounds responsible for the antioxidant action of *Vaccinium* spp. are the polyphenols, especially the flavonoids and the main hydrosoluble anthocyanins responsible for their red-blue pigments[5,6].

Many experiments have shown that a diet rich in polyphenolic compounds can be considered a healthy diet that prevent from many diseases[7]. However, the exact biological role of ingested polyphenols is not completely understood because of the many variables involved in the transition from the food product through the numerous biological changes that take place in the stomach, intestine, blood absorption and, finally, the interactions with the target site. One of the most important actions of polyphenols is their antioxidant activity, and so a first fundamental step in studying the potentially health-promoting bio-

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logical value of food plant phytochemicals is to measure of their antioxidant capacity, which can be done using a wide range of methodologies[8-11]. Many assays are used in the case of polyphenols and anthocyanins, which often give different results[12-14]. On the basis of previous studies, one general recommendation is to use more than one antioxidant assay in order to establish the most precise attribution of the antioxidant properties of a food product. Most published studies have used a chemical approach and less frequently a biological approach, but it may be more effective to use both. The ideal situation could be the use of a contemporary chemical and biological investigation for the attribution of the antioxidant activity to a phenolic-rich berry fruit. We used seven (four cell-free and three by using human cells) of the most widely used and validated antioxidant assays to identify the differentiated antioxidant activity of *Vaccinium* spp. extracts of wild and cultivated species with the aim of establishing the 50% protection (EC50) and the lowest significant concentration that still shows antioxidant activity (MSA). The extracts were from a cultivated *V. corymbosum* sample (the commonly cultivated highbush blueberry) and from a wild sample of *V. myrtillus* (the European blueberry or bilberry), which is known to have a high antioxidants content[15-17] and is widely used in the pharmaceutical market[18]. The cell-free assays were a spectrophotometry based on ABTS cation radical scavenging and three assays based on electron paramagnetic resonance (EPR) detection: the scavenging of K-nitrosodisulfonate (Fremy's salt), superoxide anion and the Fenton-generated hydroxyl radical. Two of the three biological assays were based on oxidative bursts by human blood polymorphonuclear (PMN) cells by means of the luminol amplified chemiluminescence (LACL)[19], and the third was used to investigate the effect of *Vaccinium* extracts on DNA damage (COMET), which is known to be an important sign of premature aging and can be induced by various stress agents such as oxidant free radicals[20].

Materials and Methods

Plant Material and Extracts

The plant material consisted of about 1 kg of *Vaccinium* berries belonging to two different species, obtained in the 2009 season, one cultivated and one wild. The cultivated blueberry *V. corymbosum* L., cv Berkeley (COR) was harvested at the time of commercial maturity in the experimental field of the Horticulture and Floriculture Research Centre of Lombardy, located in Vertemate con Minoprio (Como, Italy). The ripe fruit of the wild bilberry *Vaccinium myrtillus* L. (MYRT) were harvested in Val Camonica, Italy, by trained personnel of the University of Milan, Faculty of Agriculture. The berries were taken to the laboratory within 24 hours of harvesting and those of a similar size and without visual defects were selected and quickly frozen at –50°C in an air-blast tunnel. They were then cut in half, kept at –80°C overnight and then lyophilised until constant weight in order to calculate the dry matter (expressed as a percentage). After lyophilisation, the samples were powdered in a refrigerated grinder (0-2°C) and stored at –20°C until analysis. To extract phytochemicals, 200 mg of berry powder was treated at 2-4°C with 15 ml of cold H_2O, vortexed for one minute, centrifuged at 25000×g and filtrated on glass-wool. On the supernatant, the soluble solid content[21] was measured by refractometry, the titratable acidity[21] by titration with NaOH 0.1N, the total polyphenols content by Folin-Ciocalteu reaction[22] and the total anthocyanin content by differential pHmetry[23]. Moreover, the supernatant was diluted 5-fold with the cold buffer solutions used in the different antioxidant assays within three hours in an ice-bath. The reaction systems of the various assays were set up after many preliminary experiments in order to make precise comparisons of the different antioxidant tests. For each assay, a different molecular probe was used depending on the concentrations of the plant extracts. The dose *vs* effect plots had the amount of raw berry powder (μg/ml) on the x axis and the percent scavenging or oxidative protection on the y axis. The experimental approach was that of subsequently diluting the plant extract two-folds to reach the MSA *vs* the control, which was typically represented by the reaction or biological system in the absence of scavenger solutions. The exact concentrations are given for each assay (see single method descriptions). The experimental points were re-plotted on a semi-logarithmic scale (% scavenging *vs* log concentration) and linearly forced to the respective equations, and the EC50 values were determined (i.e. the amount of plant material necessary to have 50% protection).

Scavenging of ABTS$^{\bullet+}$

The free radical scavenging capacity of the various concentrations of berry powder from the COR and MYRT samples (533.4 μg/ml to 2.1

μg/ml) was initially studied using the ABTS radical cation decolorization assay, which is based on the reduction of $ABTS^{\square+}$ radicals induced by the antioxidants of the tested plant extract. The method of Re et al[24] was followed. Briefly, ABTS (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in deionised water to a concentration of 7 mM. The ABTS radical cation ($ABTS^{\square+}$) was produced by reacting ABTS solution (1 mL) with 2.45 mM potassium persulfate (10 μL) (Sigma) and leaving the mixture (stock solution) to stand in the dark at room temperature for 12-16 h to give a dark blue solution. For this study, the $ABTS^{\square+}$ solution was diluted in deionised water to an absorbance of 0.700 (± 0.02) at 734 nm, and an appropriate solvent blank reading was taken (A_B). An aliquot of the test sample (100 μL) was mixed with $ABTS^{\square+}$ solution (900 μL) in a 1 ml cuvette and the absorbance was recorded over a period of 15 min (A_E). All of the solutions were used on the day of preparation, and the percentage inhibition of $ABTS^{\square+}$ was calculated using the formula: % inhibition = $[(A_B - A_E) / A_B]\times100$.

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

EPR (electron paramagnetic resonance) is a technique that not only allows the direct detection of free radicals, but also detects the activity of molecules with antiradical activity[25,26].

The first EPR assay was performed using Fremy's salt, potassium nitrosodisulfonate, $(KSO_3)_2NO$), a persistent water-soluble radical that has been successfully used in previous experiments investigating the antioxidant potential of fruit juices[27]. A typical reaction mixture contained 2.5 μM Fremy's salt (Sigma), 0.1 M phosphate buffer, and sequential dilutions of the berry extracts (1333.4 μg/ml to 10.4 μg/ml). 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 Miniscope MS 200 EPR spectrometer (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 $100 \times (h_0\text{-}h_X)/h_0$ [%], 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.

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

The superoxide assay used the spin trapping method[26], which is based on the rapid reaction of many radicals with certain chemical acceptor molecules (spin trapping agents) to produce more stable secondary radicals. In this 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 (dimethylsulfoxide) with the addition of 18-crown-6-ether to complex K^+. The diamagnetic spin-trap nitrone, 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) was added to the reaction mixture because, under these conditions, a DMPO-OOH adduct is observed[28,29]. A typical reaction mixture contained 7.29 mM KO_2 (Sigma), 9.01 mM crown-ether (Sigma) in DMSO and 14.29 mM DMPO (Sigma), and the effects of decreasing amounts of the extracts (1333.4 μg/ml to 5.2 μg/ml) 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 Miniscope MS 200 (Magnettech, Berlin Germany), whose parameters were: field modulation 100 KHz, modulation amplitude 1500 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 scavenging capacity was expressed by means of the formula: $100\times(h_0 - h_X)/h_0$ [%], where h_0 *and* h_x are the relative heights of the highest resonance signal of the DMPO-OOH adduct spectra in a reaction mixture without and with the extract, respectively.

Fenton Reaction Model System with EPR Detection of Hydroxyl Radical ($HO^{\bullet}$)

The diamagnetic spin trap nitrone DMPO (5,5-dimethyl-1-pyrrolidine-N-oxide) was added to the Fenton reaction mixture to produce the relatively long-lived free radical product DMPO-OH, which can be easily investigated by EPR. The activity of the *Vaccinium* spp. extracts (from 666.7 μg/ml to 5.2 μg/ml) was evaluated by assessing their ability to scavenge the hydroxyl radical ($HO^{\bullet}$), the most potent active oxygen species[30]. The hydroxyl radical generated by a

standard Fenton reaction was trapped using DMPO as previously described[29] with slight modifications. The chemical system used to generate the hydroxyl radicals was: $FeSO_4 \bullet 7H_2O$ 0.31 mM (Sigma Chemicals, Co., St Louis, MO, USA), 2Na-EDTA 0.34 mM (Sigma) and H_2O_2 0.31 mM (Sigma) and DMPO 0.78 mM (Sigma). The Fenton reaction was initiated by mixing the Fe-EDTA solution with the extract or PBS (0.1 mM, pH 7.4) (control), and then adding the H_2O_2 solution. 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 one minute. 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 $HO^{\bullet}$ 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 of the DMPO-OH adduct spectra in a reaction mixture with and without the *Vaccinium* spp. extract.

Luminol Amplified Chemiluminescence (LACL), 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 Diagnostic, 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 M 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 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 means of counting in a Burker chamber (interference contrast microscopy). The effect of the extract of *Vaccinium* spp. was evaluated at concentrations ranging from 1333.4 μg/ml to 5.2 μg/ml (2.6 μg/ml for LACL-Arg), with an incubation time of the *Vaccinium* spp. extract with the cell suspension that was 15 min at 37°C.

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[19, 31,32]. 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[33,34] 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. 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), 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[19]. Briefly, 0.1 ml of PMN suspension (1×10^6 cells/ml), incubated with or without plant extract, plus 0.2 ml of 2×10^{-5} M 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 $5x10^{-7}$ M was added to reach a final volume of 1 ml. 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), and the re-

sults were expressed as percent protection in comparison with the control (samples without the addition of *Vaccinium* extract). A second series of tests on human PMNs were performed in the same way, but L-Arginine (170 μg/ml) (Sigma) was added to the medium incubating the PMNs and the plant extracts as a NO donor in order to be able to read the NO-derived peroxynitrite radical by means of LACL (LACL-Arg).

COMET Assay, Cell Culture

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

Cell Treatment

The cells were pre-treated for one hour with different dilutions of the COR and MYRT *Vaccinium* extracts (ranging from 166.7 to 20.8 μg/ml) in serum-free medium and then incubated with 250 μM *tert*-butyl-hydroperoxide for one hour at 37°C, 5% CO_2. The positive control was incubated with 250 μM *tert*-butyl-hydroperoxide for one hour at 37°C, 5% CO_2 in the absence of pre-treatment.

Single-cell Gel Electrophoresis (SCGE, COMET ASSAY)

The comet assay is a very sensitive method of detecting DNA alkali-labile sites and strand breaks in individual cells. It was carried out according to the protocols of Gminski et al[35] with minor modifications. The cells were plated in 28 mm plastic tissue culture dishes and treated as previously described. After exposure, the medium was removed and the cells were washed with 2 ml of PBS (phosphate buffered saline) and trypsinized with 300 μl trypsine for 4-5 minutes at 37°C, 5% CO_2. The trypsination was stopped with 1 ml of medium and the cell suspension was centrifuged for 5 min, at 2000 × g. The supernatant was removed, and the cells were then resuspended in 500 μl of medium and kept on ice. A sample of 2×10^4 cells/ml (counted using trypan blue method) was resuspended in 200 μl of 0.5% low melting point agarose (LMA) before being transferred onto pre-coated microscope slides with agarose 1% in PBS, and covered with a coverglass. The slides were prepared with a first layer of LMA 0.5% in PBS and a second layer of LMA 0.5%, and then stored for 10 min at 4°C to allow solidification. The coverglasses were then removed and the slides were immersed in lysis buffer (2.5 M NaCl, 100 mM Na-EDTA, 10 mM TRIS, 250 mM NaOH, plus 1% Triton X-100 and 10% DMSO added freshly on the day of the experiment, pH 10) at 4°C, for 30 min. The slides were then rinsed with neutralisation solution (0.4 M Tris, pH 7.5) and placed in a horizontal gel electrophoresis tank (Biocom) filled with ice-cold electrophoresis buffer (0.3 M NaOH, 1 mM Na-EDTA, pH 13) for 20 min on ice in the dark under alkaline conditions in order to allow the DNA supercoils to relax and express DNA single-strand breaks and alkali labile sites. Electrophoresis was conducted at 25 V and 300 mM for 20 min, followed by 5 min neutralisation with 2 ml of neutralisation solution, and fixation on ice-cold ethanol for 5 min. When the slides were dry, they were stained with 500 μl of propidium iodide solution (20 μg/ml) and analysed using a fluorescence microscope (Axioplan, Zeiss, Milan, Italy) at 25-fold magnification. At least 50-60 randomly chosen cells from each slide were analysed with an imaging software (Tritek Comet Score^{tm}, Freeware v 1.5). Cells with damaged DNA after the hydroperoxide and electrophoresis treatments would have a comet-like appearance when examined under a fluorescence microscope. The broken DNA strands would be in the "tail" region and the intact DNA would be in the "head" of the comet: the longer the comet tail, the more severe the damage. The comet parameters (tail length and % tail DNA) were calculated, and the final data were expressed as tail moment (μm), which is the product of the two parameters.

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. Each value of the COR and MYRT samples were also reciprocally compared using a *T* test. The differences were considered statistically significant when the *p* value was < 0.05. The results of the COMET assays represent the means value of the data of three independent experiments, and are expressed in the form of tail moment (TM).

Results

Vaccinium spp. Characterization

The initial chemical analysis of the blueberry samples showed significant differences in all of the tested parameters, with more dry matter and soluble solids in *V. corymbosum*, and more acidity, total polyphenols and anthocyanins in *V. myrtillus*, as shown in Table I. The anthocyanin amount found in *V. corymbosum* are in line with previous data[6], and in *V. myrtillus* interesting amounts of anthocyanins has been found (297.4 mg/100g fw), being the most phenolics present in these berry types[17]. Specifically, MYRT had 1.5 folds total polyphenols index and 2.6 folds more of total anthocyanins than COR samples.

Scavenging of ABTS•+

The initial concentration used for both *Vaccinium* species led to 100% inhibition of ABTS□+ (Figure 1A). The differences between COR and MYRT blueberries started from the second dilution: inhibition decreased to 88% in the COR samples, but remained at 100% in the MYRT sample. Successive dilutions showed further differences with inhibition remaining at 100% with the 133.3 μg/ml concentration of MYRT, but decreasing to 53% with COR. The COR blueberry extract showed the level of minimum significant activity at 16.7 μg/ml, while MYRT sample resulted active at a much lower concentration, 4.2 μg/ml.

EPR Assays

The effect on quenching of the NO radical moiety within the Fremy's salt molecule was practically the same as before (Figure 1B), with total inhibition for the first two concentrations of MYRT blueberry (1333.4 and 666.7 μg/ml) and very close to 100% inhibition for the highest concentration of the COR samples. The lowest concentration that was still significantly active was 41.7 μg/ml COR and 20.8 μg/ml MYRT. The system for the quenching evaluation of the superoxide anion (Figure 2A) was set up to give the advantage of having a more direct response related to the detection of the superoxide, a more biologically relevant free radical than Fremy's salt. Once again, the difference in scavenging patterns clearly indicated greater quenching action by the MYRT extract (Figure 2A): the three highest MYRT concentrations and only the highest COR concentration led to 100% inhibition, whereas the lowest significant action of COR was at 20.8 μg/ml and of MYRT at 10.4 μg/ml. The situation was completely different in the EPR assay in the Fenton system (Figure 2B), with a significant difference between COR and MYRT only at the highest concentration and no reciprocal significance for any subsequent concentrations. This assay showed a progressive scavenging decrease at subsequent dilutions with COR reaching the minimal significant action at a concentration of 20.8 μg/ml and MYRT at a concentration of 83.3 μg/ml.

LACL Assay

The biological assay was performed by monitoring the control of oxidative bursts in human PMN cells, starting from a concentration of 1333.4 μg/ml (Figure 3A). The trend in inhibition decreased with successive dilutions, and thus confirmed the ABTS, Fremy, superoxide and hydroxyl assays. The first four dilutions of the MYRT berries led to more than 90% inhibition, as did the first three COR samples. The successive dilutions showed a greater significant action in MYRT extracts than in COR ones. The minimal significant active concentration useful in controlling oxidative stress was 20.8 μg/ml in COR and 10.4 μg/ml in MYRT. Similar results were found in the LACL-Arg assay, which showed not less than 90% scavenging of the first five MYRT dilutions (Figure 3B), the significant higher scavenging action at the intermediate doses, and a minimum significant active dose of 5.2 μg/ml. The COR extracts (first three dilutions

Table I. General quality chemical parameters measured on *Vaccinium* (COR: *V. corymbosum*, MYRT: *V. myrtillus*). Different letters in each row indicate significant difference ($p < 0.05$).

	COR	MYRT	(MYRT/COR)
Dry matter (%)	15.9 ± 2.1[a]	12.4 ± 0.3[b]	0.78
Soluble solids (°Bx)	9.8 ± 0.3[a]	7.7 ± 0.5[b]	0.79
Titratable acidity (mEq %)	8.6 ± 2.2[b]	18.3 ± 3.5[a]	2.13
Total polyphenols (mg/100 g fw)	225.6 ± 5.8[b]	339.3 ± 24.7[a]	1.50
Total anthocyanins (mg/100 g fw)	114.3 ± 7.8[b]	297.4 ± 57.0[a]	2.60

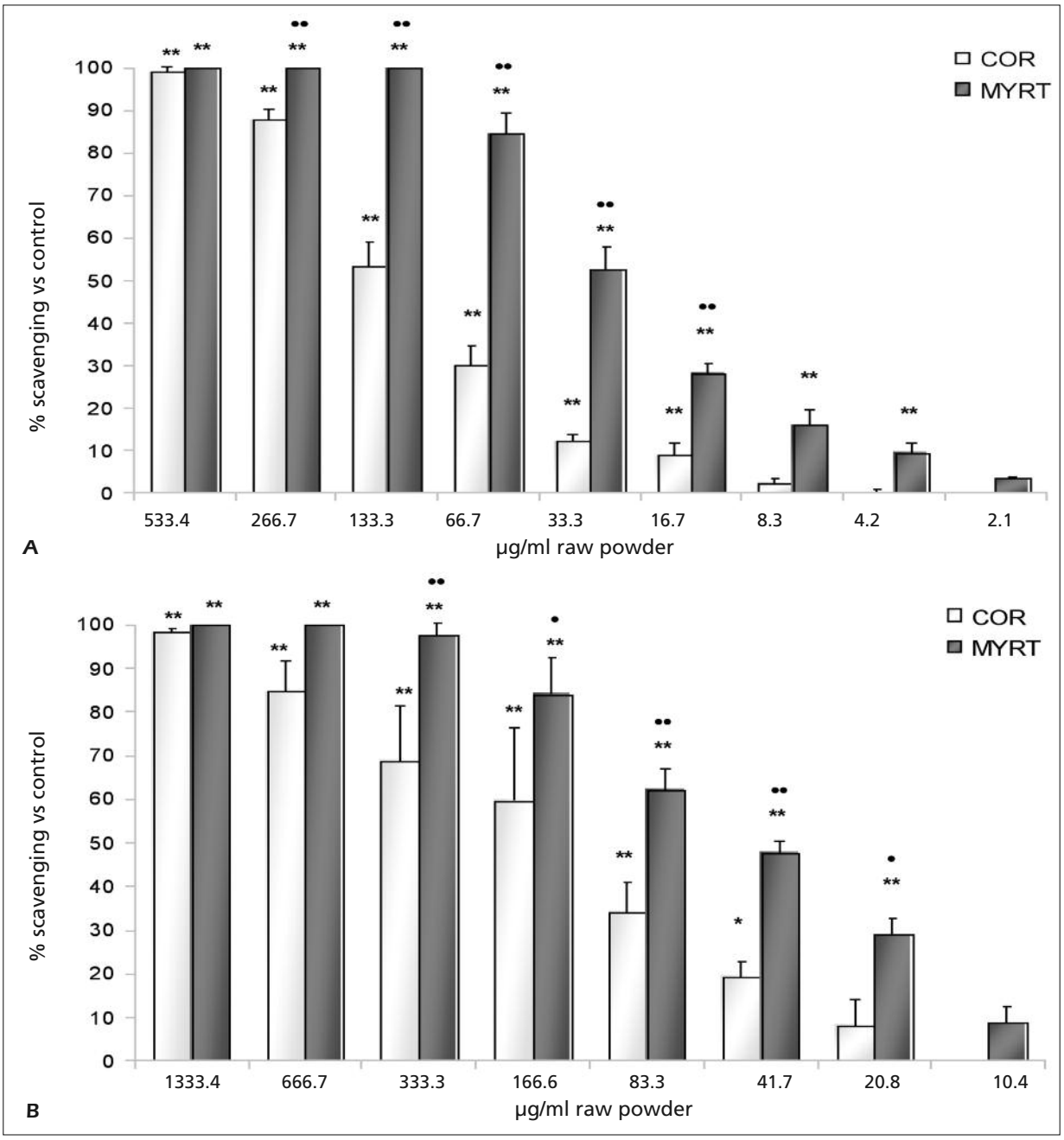

Figure 1. ABTS ***(A)*** and Fremy's salt ***(B)*** on raw powder extracts of *V. corymbosum* (COR) and *V. myrtillus* (MYRT). ***$p < 0.05$, $p < 0.01$ vs control, 0 µg/ml(ANOVA + Dunnet's test); •••$p < 0.05$, $p < 0.01$ vs *V. corymbosum* at the same dose (*t*-test).

higher than 90% scavenging, minimum active dose 20.8 µg/ml) were also active, but less than MYRT, significantly lower than MYRT in the range 166.6-10.4 µg/ml (Figure 3B).

Single-Cell Gel Electrophoresis (SCGE, COMET ASSAY)

Reactive oxygen species (ROS) can cause oxidative DNA damage, damage proteins and lipids, and induce DNA base oxidation. The activation of antioxidant responses can reduce oxidative DNA damage by neutralising ROS formation. In this study, we used an alkaline comet assay to demonstrate the induction of oxidative DNA damage in A549 cells by *tert*-butylhydroperoxide, and the role of the two extracts in protecting against this damage. Figure 4 shows the results of the alkaline comet assay, pH > 13,

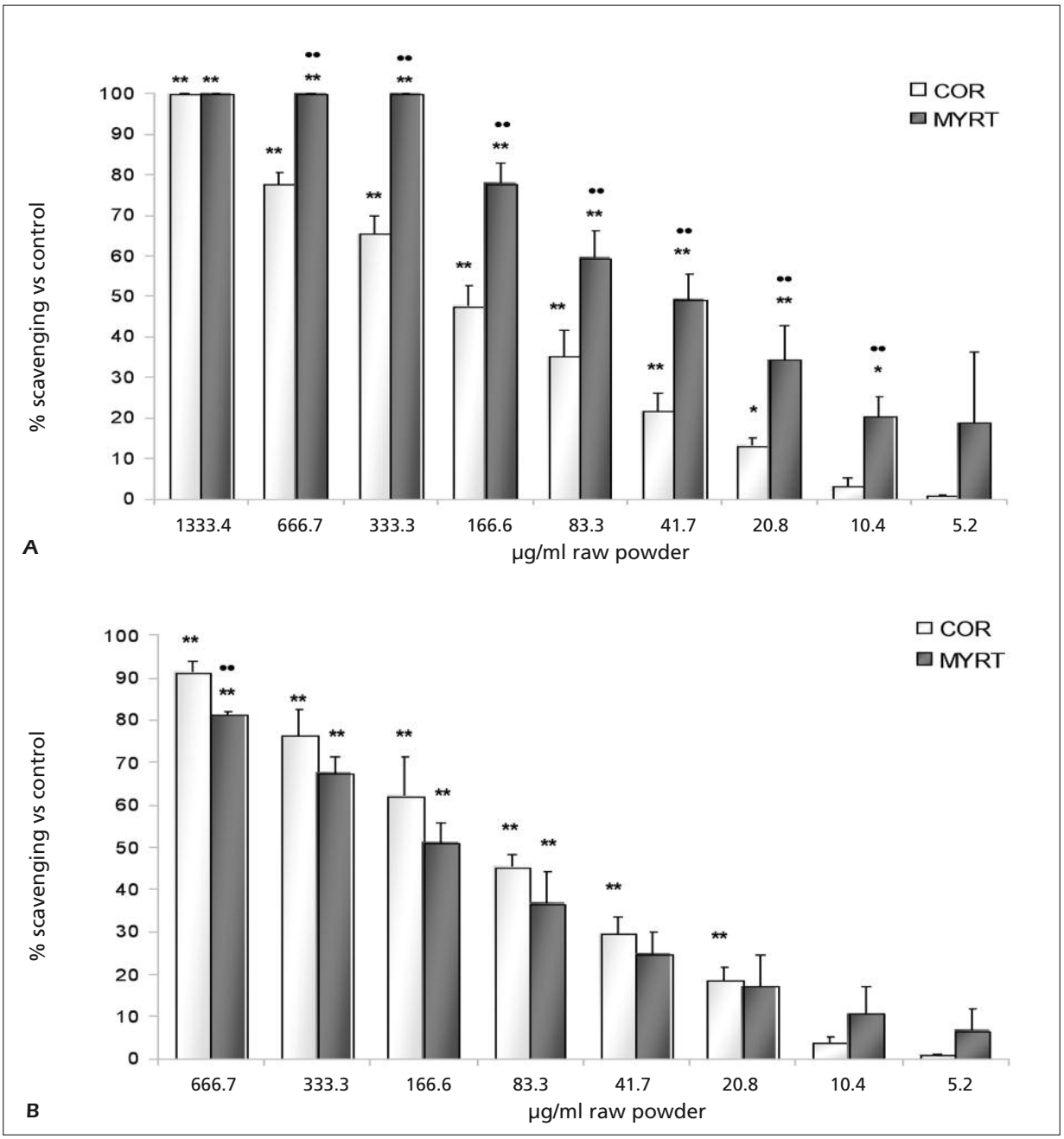

Figure 2. Superoxide anion ***(A)*** and hydroxyl radical scavenging ***(B)*** on raw powder extracts of *V. corymbosum* (COR) and *V. myrtillus* (MYRT). *, **$p < 0.05$, $p < 0.01$ vs control, 0 μg/ml (ANOVA + Dunnet's test); ••$p < 0.01$ vs *V. corymbosum* at the same dose (*t*-test).

with the tail moment (TM) parameter. The cells exposed only to 250 μM *tert*-butylhydroperoxide for one hour showed damage, whereas the protective effect of pre-treatment with COR and MYRT *Vaccinium* extracts is clear. The cells exposed to the lowest dilution (20.8 μg/ml) of MYRT extract showed a significant decrease in damage, followed by the other dilutions and with absolute protection at the starting dilution ($p < 0.01$, 166.6 μg/ml), whereas COR *Vaccinium* extract reduced DNA damage, but this was statistically significant ($p < 0.05$) only at 166.6 and 83.3 μg/ml, clearly demonstrating a minor protection in comparison with MYRT bilberry extracts.

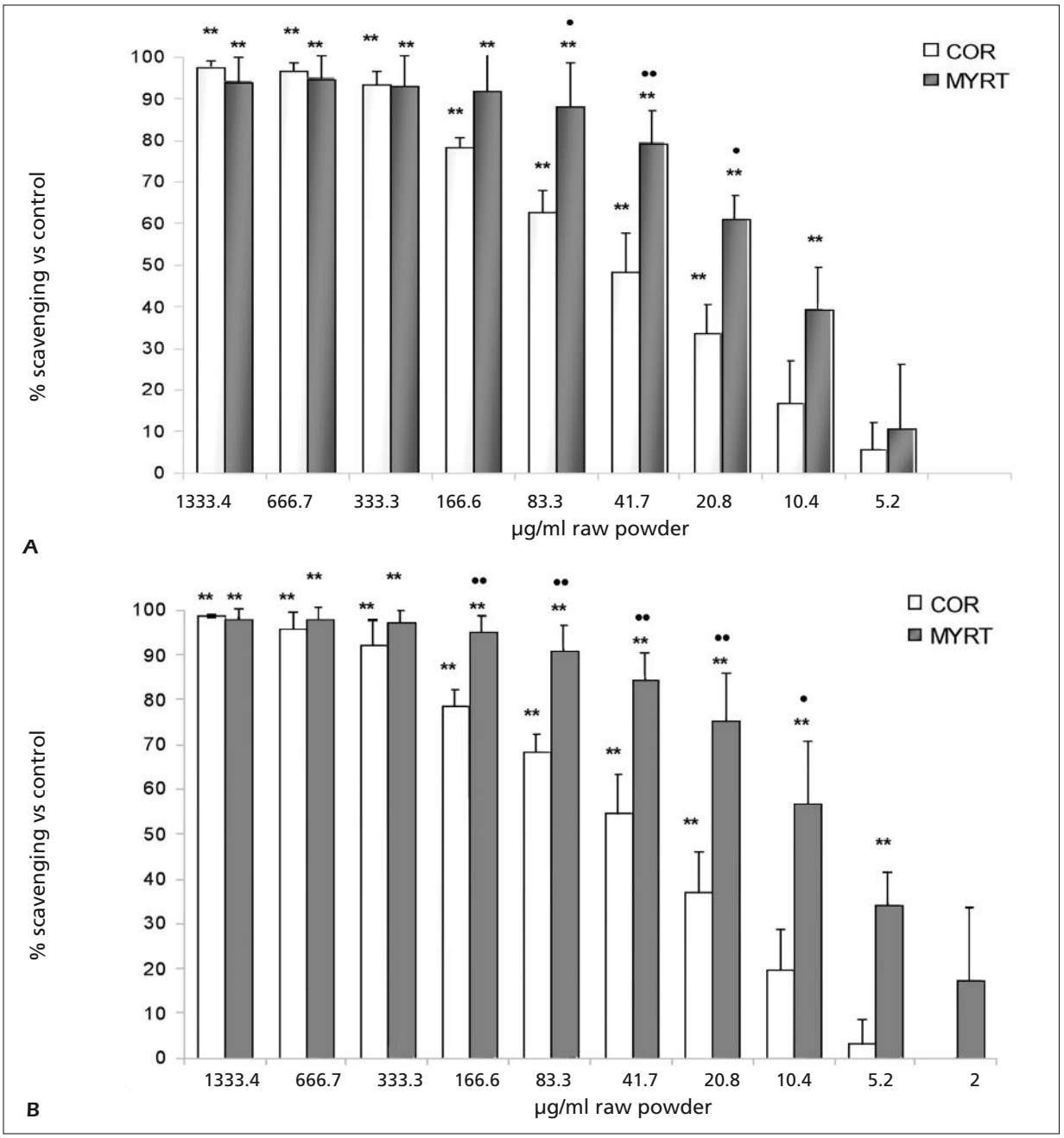

Figure 3. LACL/flmp ***(A)*** and LACL/flmp + L-Arg ***(B)*** on raw powder extracts of *V. corymbosum* (COR) and *V. myrtillus* (MYRT). $^{**}p < 0.01$ vs control, 0 µg/ml (ANOVA + Dunnet's test); •, •• $p < 0.05$, $p < 0.01$ vs *V. corymbosum* at the same dose (*t*-test).

Discussion

In this study, the oxidant free radical concentrations were adjusted in order to look for a relationship between their detection in the different assays and the plant extract concentrations. Similar concentrations were used for each assay to explore the responses between 100% inhibition and the lowest concentration still capable of giving a significant response against the control sample. The different cell-free assays showed different sensitivity, given by the lowest probe final concentration (see Materials and Methods), as follows: Fremy's salt > hydroxyl radical > ABTS > superoxide. The situation of the LACL and COMET assays is different, due to the intrin-

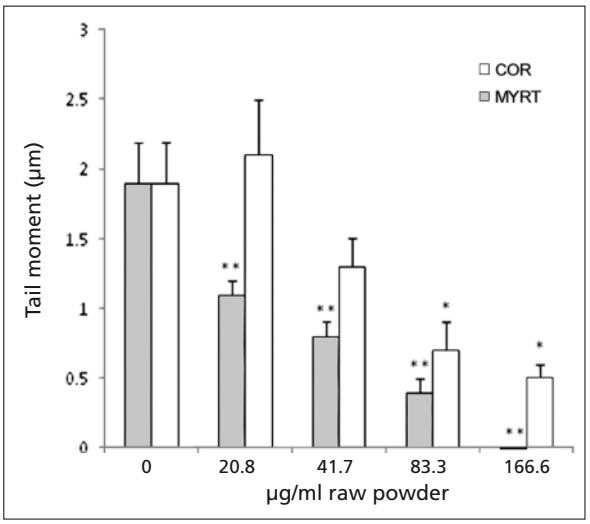

Figure 4. Comet assay on raw powder extracts of *V. corymbosum* (COR) and *V. myrtillus* (MYRT). *, **$p < 0.05$, $p < 0.01$ vs control (0 μg/ml) (ANOVA + Dunnet's test).

sic variability of biological samples. The assays were compared using the EC50 of the determinations of the semilogarithmic slopes dose *vs* effect in the linear portion of the reaction plots (Table II). This approach allows a more precise comparison: the lower the EC50, the higher the reaction response in relations to the active extract concentration. The lowest EC50 in COR sample was shown by the LACL-Arg data, and the highest by the Fremy's salt assay. The former was confirmed for the MYRT extract for the lowest amount, but not for the highest, which was in the hydroxyl radical assay (Table II). The EC50 data show that LACL assays are more sensitive than the others, confirming previous finding using eggplant extracts[36]. The percent scavenging and EC50 data based on ABTS, superoxide and Fremy's salt scavenging are related to the difference in polyphenol and anthocyanin content between MYRT and COR. Moreover, the LACL and COMET assays (considered to be of the greatest biological relevance) also showed about the same response, with only the assay of the hydroxyl radical giving a different result.

Table II. EC 50 values measured from the plots dose vs effect of *Vaccinium* extracts assayed in different antioxidant assays (COR: *V. corymbosum*, MYRT: *V. myrtillus*).

	EC50 (µg/ml dw)		(MYRT/COR)$^{-1}$
	COR	MYRT	
ABTS	83.52	26.37	3.17
Fremy'salt	147.19	52.29	2.83
Superoxide anion	140.51	41.12	3.42
Hydroxyl radical	93.26	133.79	0.70
LACL/flmp	47.07	17.64	2.67
LACL/flmp + Arg	44.83	8.16	5.49
Mean			3.04

The interesting fact is that the action of the MYRT sample was stronger than that of COR in all of the assays, except for hydroxyl radical, as shown by the reciprocal values of the EC50 ratios (Table II): the highest ratio between the EC50 of MYRT and COR samples was found in LACL-Arg, followed by superoxide, ABTS, Fremy's salt and LACL. This suggest that anthocyanins play an important role, 2.6 fold higher in MYRT than in COR (Table I), a value close to the average activity ratio (3.04, Table II) and that the role of total polyphenols is less (ratio of total polyphenols amount MYRT/COR at 1.5, Table I). The hydroxyl radical results show a different trend, with the lowest EC50 ratio between MYRT and COR (0.70, Table II). It is clear that antioxidant substances other than polyphenols and anthocyanins are involved in the scavenging of hydroxyl radical. This confirms previous observations, showing that cells have not adapted an enzymatic factor against the hydroxyl radical, because of its very high reactivity, which is close to the diffusion rate[37]. A number of previous studies have demonstrated that unusual antioxidant molecules are active against the hydroxyl radical such as simple sugars[38-40] and carboxylic acids[41-42]. It is worth pointing out that the radical concentration used in the superoxide and hydroxyl radical quenching tests is in the same order as that found in living human cells[43], so that they can be considered biochemically relevant. In LACL assays, both superoxide and hydroxyl radical are produced during the neutrophil bursts, with the substantial production of NO radicals in the LACL-Arg assay. The MYRT extracts were much more active than the COR extracts, and so it can be inferred from the present data that they had a prevalent action on superoxide, which is a precursor of hydroxyl radicals and an inducer of instantaneous peroxynitrite synthesis. Hydroxyl radicals (assayed in an isolated Fenton system) do not express the strong difference between MYRT and COR extracts. The trend of a greater MYRT activity in comparison with COR was supported by the COMET assay: the highest used dilution (20.8 μg/ml) was efficient in controlling DNA damage in the MYRT extract, but not in

the COR extract, which remained significantly efficient only at 83.3 μg/ml, two dilutions higher (Figure 4). In this case, polyphenols and especially anthocyanins seem to be fundamental in protecting against peroxyl radical-induced DNA damage. The data concerning the MSA (Table III) arise further considerations. The highest reactivity, given by the lowest concentration value, was in the ABTS assay for both berry types. On the other hand, the highest values for COR and MYRT, respectively were found in the Fremy and hydroxyl assays. ABTS and LACL-Arg showed the highest ratio of MYRT *vs* COR activity, with uniform values for the other assays except the hydroxyl radical assay. From an exclusively theoretical point of view, the dosage of raw blueberry can be calculated in order to evaluate the biological relevance of all the assays, particularly LACL: if a neutrophil content of 40×10^9/ml is considered against 10^6 cells/ml in the assay, to control 50% of the potential oxidative bursts in the human body requires 11.8 (11.3 with L-Arg) g fresh weight of COR and 5.7 (2.6 for L-Arg) g of MYRT berries (Table IV). Moreover, 5.2 g and 3.4 g are needed if the calculation is based on the minimum berry dose still showing significant activity in LACL (Table IV). Lower amounts resulted from LACL-Arg assay: 3.3 and 2.4 g for COR and MYRT, respectively. These can be considered as very interesting amounts given the low bioavailability of anthocyanins, the main phenolic components of *Vaccinium* berries. The absorption of raw anthocyanins was about 1% or less, but that can be increased significantly by more than 50% if the anthocyanidin degradation compounds such as phenolic acids and aldehydes are considered[44,45]. This degradation can be favoured by the pH conditions of the reaction solutions in the assays, all of which have buffers at a physiological pH of about 7, which allows the conversion of the anthocyanin structure form from the more acid-stable flavylium coloured ionic form to the less stable colourless hemi-acetal forms[12]. If the same calculation is made on the basis of a hypothetical concentration of 10^6 cells/ml in the cell-free assays in a simulated way, the resulting amounts are all in a range that is close to the real situation: a range in fresh berries amount of 8.5-43.2 g is obtained from EC50 values, with 1.4-26.9 g for the minimum active dose (Table IV), this further confirming the optimal choice of the assays. Obviously, all of the required amounts are lower in the case of MYRT with the exception of the hydroxyl radical data. It is possible that future research into fruit quality will consider the antioxidant content of *Vaccinium* spp., but further work has to be done with the main objective to give a more precise attribution of antioxidant potential to the different phytochemicals in *Vaccinium spp*., by using purified extracts on cell-free assays supported by assays using living cells.

Table III. Minimum significant active concentrations (MSA) measured from the plots dose vs effect of *Vaccinium* extracts assayed in different antioxidant assays (COR: *V. corymbosum*, MYRT: *V. myrtillus*).

	MSA (µg/ml dw)		(MYRT/COR)$^{-1}$
	COR	MYRT	
ABTS	16.7	4.2	4
Fremy'salt	41.7	20.8	2
Superoxide anion	20.8	10.4	2
Hydroxyl radical	20.8	83.3	0.2
LACL/flmp	20.8	10.4	2
LACL/flmp + Arg	20.8	5.2	4
Mean			2.4

Table IV. Theoretical dose of free radicals inhibition in 40×10^9 neutrophils starting from 10^6 cells per ml for each assay. In ABTS, Fremy's salt, superoxide anion and hydroxyl radical the dose is simulated (COR: *V. corymbosum*, MYRT: *V. myrtillus*).

	From EC50				From minimum significant active conc.			
	Dry weight (g)		Fresh weight (g)		Dry weight (g)		Fresh weight (g)	
	COR	MYRT	COR	MYRT	COR	MYRT	COR	MYRT
ABTS	3.3	1.1	21.0	8.5	0.7	0.2	4.2	1.4
Fremy'salt	5.9	2.1	37.0	16.9	1.7	0.8	10.5	6.7
Superoxide anion	5.6	1.6	35.3	13.3	0.8	0.4	5.2	3.4
Hydroxyl radical	3.7	5.4	23.5	43.2	0.8	3.3	5.2	26.9
LACL/flmp	1.9	0.7	11.8	5.7	0.8	0.4	5.2	3.4
LACL/flmp + Arg	1.8	0.3	11.3	2.6	0.5	0.3	3.3	2.4

Conclusions

Seven different assays (four cell-free and three using human cells) were compared with the aim of determining the antioxidant activity of cultivated and wild *Vaccinium*. Most of the assays gave a response in line with anthocyanin content, with the sole exception of the hydroxyl radical assay, which was less specific and probably linked to antioxidants other than anthocyanins and polyphenols. The difference between the cultivated and wild samples (expressed by the ratio between each antioxidant parameters) was comparable in the cell-free and biological assays, with the exception of the isolated hydroxyl radical assay, which showed a different trend. Furthermore, the biggest difference in the activity of wild *vs* cultivated berry samples has found in LACL-Arg and ABTS, and are in line with the data from COMET assay. Finally, it is suggested that anthocyanins may undergo decomposition under assay conditions, mainly due to the pH value of the assays, without losing their antioxidant potency.

Acknowledgements

Special thanks to colleagues for obtaining the Vaccinium fruit. The cultivated fruit come from the School of Agricultural Sciences of Minoprio Foundation, Como, Italy, and the wild fruit have been picked by Dr. Giuseppe Granelli, of the University of Milan, Department of Crop Science, Italy.

Conflict of Interest

The Authors declare no conflict of interest, being the present study exclusively made and sustained with public fundings.

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