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Food & Function

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Journal:	*Food & Function*
Manuscript ID	Draft
Article Type:	Paper
Date Submitted by the Author:	n/a
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Food & Function

2014 Impact Factor: 2.791
www.rsc.org/foodfunction

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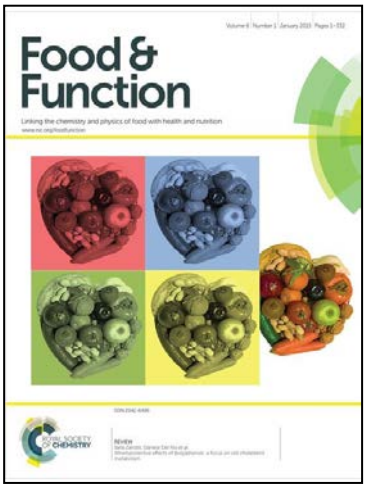

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Best wishes,

Philippa Ross	Professor Kevin Croft
Executive Editor	Editor-in-Chief

Dear Editor Prof. Isabel Ferreira, Editor of *Food & Function*,

Please find enclosed the electronic version of the manuscript entitled 'Blue honeysuckle fruit (*Lonicera caerulea* L.) from eastern Russia: phenolic composition, nutritional value and biological activities of its polar extracts'.

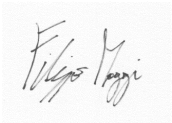
Nowadays, blue honeysuckle takes place in the group of the so-called 'superfoods' that are much appreciated for health benefits in addition to their nutritional values. In this work we conducted a comprehensive chemical analysis of blue honeysuckle spontaneously growing in eastern Russia by analysing the phenolic composition as well as the macro- and micro-nutrients. These analyses confirmed the good nutritional value of its berries. Furthermore, we assayed the *in vitro* biological activities of berries polar extracts, namely the antioxidant, antimicrobial, cytotoxic on tumor cells, wound healing and immunomodulatory effects. Some activities previously reported in literature were confirmed while others revealed to be negligible. In this regard, the novelty was represented by the likely immunosuppressive effect of honeysuckle aqueous extract on human lymphocytes that raises a potential risk related to the consumption of these berries and their derivative products.

We believe that our findings will be of interest to many readers of your journal. This research has not been published before and is not being considered for publication elsewhere. Finally, the authors declare that there are no conflicts of interest.

I would be grateful if you would consider the manuscript for publication in *Food & Function*.

I look forward to hearing from you.

Yours Sincerely,



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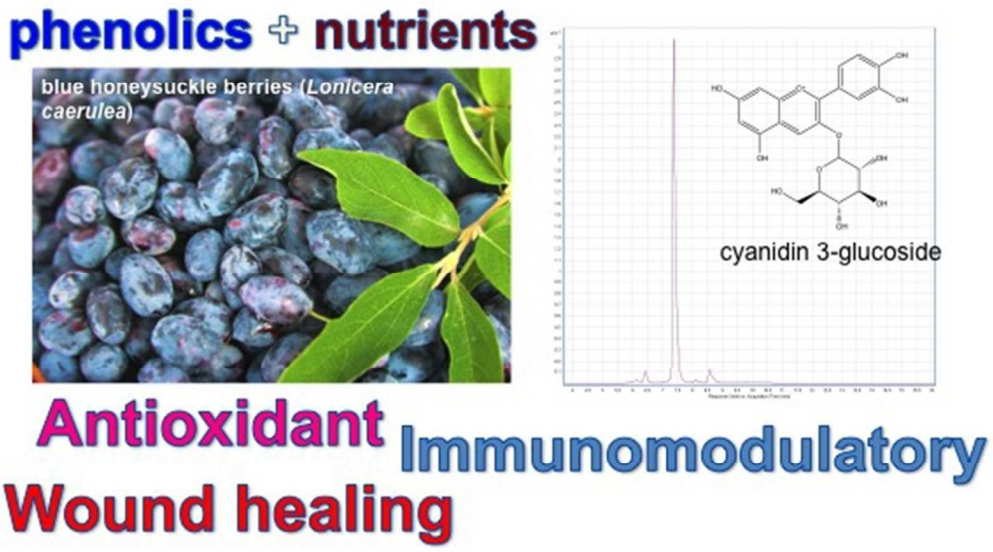

201x115mm (72 x 72 DPI)

Food & Function

ARTICLE

Blue honeysuckle fruit (*Lonicera caerulea* L.) from eastern Russia: phenolic composition, nutritional value and biological activities of its polar extracts

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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In the present work we conducted a comprehensive chemical analysis on blue honeysuckle (*Lonicera caerulea*) spontaneously growing in eastern Russia by determining the content of healthy phenolic components, and macro- and micro-nutrients in berries. HPLC-DAD-ESI/MS analysis showed cyanidin-3-glucoside as the major constituent among phenolics, while nutritional analysis revealed fibre, protein, calcium and magnesium as the most important macro- and micronutrients, respectively. Fatty acid composition was dominated by polyunsaturated fatty acids, being linoleic acid the most abundant. Furthermore, we evaluated several in vitro biological activities such as antioxidant, antimicrobial, antiproliferative, wound healing and immunomodulatory effects of blue honeysuckle aqueous and ethanolic extracts that often are incorporated in food and nutraceutical preparations. While the fruit extracts revealed to be potent radical scavengers with important inhibition on ABTS radical (IC_{50} comparable to that of Trolox), thus confirming literature data, their inhibitory effects against microbial pathogens and tumor cell lines (A375 and MDA-MB 231) were negligible. The fruit aqueous extract did not show toxicity on human fibroblasts, but 24 h treatment with 150-200 µg mL^{-1} of extract slightly enhanced the cell migration when tested by scratched wound assay. Worthy of mention was the inhibitory effect displayed by blue honeysuckle fruit aqueous extract on human lymphocytes by PBMC proliferation assay that was probably dependent on its ability of counteract or compete with some costimulatory signals.

Introduction

Lonicera caerulea L. also called blue honeysuckle is a traditional crop belonging to the *Caprifoliaceae* family. This long-lived and deciduous shrub is one of the 180 species of the genus Lonicera and native to the Northern Hemisphere. Notably, the variety *L. caerulea* var. *kamtschatica* is spontaneous and cultivated in eastern Russia up to the Kamchatka peninsula. The plant is up to 2 m in height and can grow up to the age of 25-30 years. Its older branches have a yellowish-brown to reddish flaking bark. Flowers are endowed with bracteoles that are fused into a tubular cupule enclosing the ovary, which form a succulent fruit.[1] Blue honeysuckle fruit is a dark purple to blue, oval few-seeded berry with a bitter to sweet taste, ripening between May and June. When cultivated, it can measure 2 cm in length and more than 1.5 g in weight. Fruit yield varies between 2 and 3 kg/plant.[2] The main peculiarity of blue honeysuckle is its outstanding frost resistance, since it is able to survive very low temperatures (up to - 46 °C) without being damaged. For this reason its fruits are commercialized and consumed especially in north hemisphere countries such as Russia.[2] Their main uses as a food regard mainly the preparation of jams, jellies, wine, candies, gelatin, snacks, as well as that of making tea, juice, canned and frozen fruits.[3] The honeysuckle berries were also intensively used in the traditional medicine of Russia, China and Japan for centuries. Notably, fresh and fruit juice were used to treat stomachaches and tonsillitis as well as infectious diseases.[4]

Nowadays, blue honeysuckle takes place in the group of the so-called 'superfoods' that are much appreciated for health benefits in addition to their nutritional values. The berries contain high levels of vitamin C, anthocyanins, phenolic acids and flavanols.[2] The amount of phenolic compounds present is dependent on the maturity, genetic diversity, climate, processing and storage conditions. Vitamin B, magnesium, phosphorus, calcium and potassium are minor constituents reported in the berries. Therefore some cultivars are selected on the base of research for commercial

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production in several countries such as Russia, Poland, Romania, Czech Republic, China and also US.

Recent studies have supported the use of honeysuckle berries in the treatment of atherosclerosis, hypertension, gastrointestinal disorders, diabetes, cardiovascular diseases, bacterial infection and UV-caused skin damage.[5-7]

The main aim of this work was to analyse the phenolic composition together with the content of macro- and micronutrients of blue honeysuckle berries from spontaneous plants growing in eastern Russia in order to ascertain their influence on diet. Given the therapeutic properties claimed for these fruits and their derivatives we have assayed the polar extracts of honeysuckle berries for some biological activities, namely antimicrobial, antioxidant, antiproliferative, wound healing and immunomodulatory in order to provide scientific evidences for their use as a functional food and in cosmeceutics.

Experimental

Plant material

The berries of *L. caerulea* var. *kamtschatica* were collected from three plants spontaneously growing in the taiga near Khabarovsk (Russia, about 80 m above sea level), close to China borders, in June 2014. A plant herbarium specimen was collected, authenticated and deposited in the Herbarium Universitatis Camerinensis (included in the online edition of Index Herbariorum by the New York Botanical Garden: http://sweetgum.nybg.org/ih/) of School of Biosciences and Veterinary Medicine (University of Camerino, Italy) under the accession codex CAME 27648; it is also available in the anArchive system (http://www.anarchive.it). Before analysis honeysuckle berries were stored in a falcon tube at -20 °C. For ascorbic acid determination, the fresh sample was analysed within three days after its collection to minimise compound degradation.

Reagents and standards

The analytical standards were purchased from Sigma-Aldrich (Milano, Italy). The stock standard solution was prepared by dissolving 10 mg of the analyte in 10 mL of methanol (or water for ascorbic acid) and stored in a glass-stoppered bottle at 4°C in the dark. Standard working solutions, at various concentrations, were daily prepared by appropriate dilution of aliquots of the stock solutions in water. HPLC-grade methanol and acetonitrile were purchased from Sigma-Aldrich (Milan, Italy), while HPLC-grade formic acid 99-100% was bought from J.T. Baker B.V. (Deventer, Holland). For sample preparation and chromatographic analysis, deionized water ≥ 18 MΩ cm^{-1} resistivity purified with a Milli-Q system (Millipore, Bedford, USA) was used. All solvents and solutions were filtered through a 0.45-µm PTFE filter from Supelco (Bellefonte, PA, USA) before use. ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and Folin-Denis' reagent were purchased from Sigma Aldrich (Milan, Italy), anhydrous FeCl3 from J.T. Baker D.V. (Deventer, Holland) and NaCO3 from Carlo Erba reagents (Milan, Italy). Ethanol, gallic acid and activated MnO_2 were purchased from Fluka (Buchs, Switzerland). Flat-bottomed 96-well microplates (FALCON 96, BD Biosciences) were used to do the colorimetric measurements with a FluoSTAR omega spectrophotometer, BMG Labtech (Offenburg, Germany). All solvents and solutions were filtered through a 0.45-µm PTFE filter from Supelco (Bellefonte, PA, USA) before use.

Fruit extraction

Polyphenols and chlorogenic acids

Five g of freeze-dried blue honeysuckle berries were extracted with 50 mL of two different solvents, i.e. distilled water and ethanol. After 24 h extraction under magnetic stirrer, the extract was centrifuged for 15 min at 9000 rpm at 8°C. The supernatant was dried with rotavapor, freeze-dried and stored in freezer. The samples were prepared by re-dissolving 10 mg of each extract with 1 mL of methanol. The sample solutions were filtered through a 0.45 µm pore size nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA) before injection into HPLC-DAD. Each sample was analyzed in triplicate. The above extracts were also used in biological assays.

Anthocyanins

The freeze-dried berries (3 g) were ground and the obtained powder was extracted three times with 100 mL of 70% EtOH adjusted to pH 1.8 by HCOOH, over a 1 h period under stirring, and centrifuged by Hermle LaborTechnik (5000 rpm × 5 min). The three extracts were combined up to a final volume of 500 mL. The obtained sample was analyzed by HPLC- DAD-MS.

Ascorbic acid

Sample preparation procedure was performed according to Caprioli *et al.*[8] Before extraction, fresh material was homogenized with 10 mL of liquid nitrogen for 5 min and reduced into a powder in a mortar. Five g of each sample were extracted for 4 h with 25 µL of extractant solution, containing water with 5% MPA (meta-phosphoric acid), kept in the dark. The extraction was carried out in triplicate and the obtained solutions were centrifuged at 5000 rpm for 20 min and then filtered before HPLC-MS analysis. In order to estimate the water content, fresh material was left in a stove at 133 °C for 24 h; moisture content was determined by the weighing of the samples of each collection before and after drying.

HPLC Analyses

Polyphenols and chlorogenic acids

HPLC-DAD studies were performed using a Hewlett-Packard HP-1090 Series II (Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a model 1046A HP photodiode array detector (DAD) following a previous method developed in our laboratory.[9] Briefly, the chromatographic separation was accomplished on a Synergi Polar-RP (4.6 mm x 150 mm, 4 µm) analytical column from Phenomenex (Chesire, UK). The column was preceded by a security cartridge. The mobile phase for HPLC-DAD (diode array detector) analyses was a mixture of (A) water and (B) methanol, flowing at 0.7 mL min^{-1} in isocratic conditions: 60% A, 40% B. The injection volume was 5 µl. UV spectra were recorded in the range 210-350 nm for eleven compounds, where 210 nm was used for quantification of shikimic acid, gallic acid, (+)-catechin hydrate, (-)-epicatechin, 310 nm for p-coumaric acid and trans-resveratrol, 325 nm for caffeic and *trans*-ferulic

acids, 3-O-caffeoylquinic, 5-O-caffeoylquinic and 3,5-di-O-caffeoylquinic acids.

Anthocyanins

The analysis was carried out using a HP-1100 liquid chromatograph equipped with a DAD detector and coupled with HP 6200 series MS/TOF with ESI interface (Agilent Technologies, USA). The positive ionization mode was applied to detect the anthocyanins using different fragmentors, in the range 80–200 V. The column was a Luna RP 18 (5 µm; 150 x 3 mm i.d.) from Phenomenex. The mobile phase was (A) water pH 2.0 acidified by HCOOH and (B) acetonitrile. The following multistep linear gradient was applied: from 95% to 85% of A in 3 min, 11 min to reach 75% A, then 6 min to arrive at 65% A, 2 min to reach 100% B. Total time of analysis was 22 min, flow rate 0.4 mL min^{-1} and oven temperature 26 ± 0.5 °C. Quantitative evaluation of anthocyanins was performed using four-point regression curves (r^2 = 0.9998) of keracyanin (cyanidin-3-O-rutinoside) as reference compound, calculated at 520 nm in the linearity range 0-1.7 µg g^{-1}. All the anthocyanins were expressed in keracyanin (MW 595), applying the correction factor for the molecular weight.

Ascorbic acid

The analysis of the ascorbic acid was achieved on a Synergi Polar-RP C18 (4.6 mm x 150 mm, 4 µm) analytical column from Phenomenex (Chesire, UK) and performed according to Caprioli *et al*[10]. Briefly, the mobile phases for HPLC-ESI-MS (triple quadrupole) analyses were water with 0.1% formic acid (95) and methanol with 0.1% formic acid (5) at a flow rate of 0.7 mL min^{-1} in isocratic conditions. HPLC–MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an ESI source operating in negative ionisation mode. The injection volume was 5 µl. The temperature of the column was 30 °C and the temperature of the drying gas in the ionisation source was 350 °C. The gas flow was 13 L min^{-1}, the nebulizer pressure was 60 psi and the capillary voltage was 4000 V (negative and positive). Quantifications were performed by analyzing in selected ion monitoring (SIM) mode the ions m/z 175.1 $[M-H]^-$ for ascorbic acid.

Proximate analysis and mineral content

Blue honeysuckle berries were analysed for chemical composition (moisture, protein, fat, dietary fiber, carbohydrates and ash).[10] The moisture content was calculated by oven drying the sample to a constant weight (24 h, 133 °C). The crude protein content of the samples was estimated by the Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Dietary fiber content was determined by a gravimetric method after acidic hydrolysis of the samples. Total carbohydrates were calculated by difference. Total energy was calculated according to the following equations[11]:

Energy (kcal) = 4 X (g protein + g carbohydrate) + 9 X (g lipid)

Blue honeysuckle berries were also used to determine the macro-elements (K and Ca) and micro-elements (Si and Cu) concentrations.[10] One g of each sample powder was digested with HNO3 and HClO4 in ratio 4:1 for 15 min at 350°C till clear solution was obtained. Desired volume of double distilled water was added to the digested and cooled samples. Mineral contents in all the digested samples were analyzed through ICP-MS (Agilent 7500s, Agilent Technologies, Waldbronn, Germany). The results, expressed as weight percentage on a fresh weight basis, were obtained from triplicate measurements of each sample.

Fatty acid composition

Ethanol–hexane lipid extracts were obtained as previously reported.[12] Briefly, berries (5 g each) were cut and homogenized with hexane (10 mL), ethanol (5 mL), and water (5 mL), by using a disperser (Ultra-Turrax IKA 25 Basic) to obtain a slurry which was centrifuged. The upper liquid phase was transferred. The treatment was repeated two more times adding each time 10 mL of hexane to the aqueous phase. The final organic extract was dried and finally dissolved with hexane and stored at −20 °C. The extraction was performed in triplicate obtaining a relative standard deviation of 3.5 %. Each ethanol–hexane lipid extract (5 mg) was subjected to basic transmethylation using potassium hydroxide in methanol, following Ichihara's procedure.[13] Fatty acid methyl esters (FAMEs; Supelco, Bellefonte, PA, USA) hexane solutions (1 mL) were analyzed by Agilent Technologies 6850 series II gas chromatograph equipped with a flame ionization detector (GC–FID). FAMEs identification was performed analyzing the standard mixture "37 component FAME Mix" from Supelco. For calculating the relative fatty acid percentages on a weight basis, the area values obtained from the GC–FID analysis of FAMEs, corrected with the theoretical response factors have been used. The carrier gas used for the GC–FID analysis was hydrogen at a constant flow of 3.7 mL min^{-1}. The injector was held at 260 °C. The capillary chromatographic column was a 30 m DB-225 (ID, 0.32 mm; film thickness, 0.25 µm; Agilent Technologies). The injector temperature was 260 °C. The split ratio was 1:30. The oven temperature was held at 60 °C for 3 min, then programmed to increase to 220 °C at 20 °C min^{-1}, and held at 220 °C for 8 min. The detector was held at 250 °C.

Antioxidant activity

Determination of total polyphenolic content (Folin–Ciocalteu method)

The total content of polyphenolics was determined by a colorimetric method described by Singleton and Rossi.[14] The two freeze-dried blue honeysuckle fruit extracts were re-dissolved with 1 mL of methanol and 100 µL of each extract was introduced into test tubes, then 150 µL of Folin Ciocalteu reagent (1 mL Folin-Denis' reagent in 4 mL H_2O) was added. The plate was incubated for 10 min at 37 °C. Next, 50 µL of a saturated Na_2CO_3 solution in H_20 was added to each well and the plate was incubated for other 10 min. The absorbance was measured at 765 nm. Total phenolic content was expressed as gallic acid equivalents (GAE) per g of dry weight of fruit extract. The results were expressed as average of three measurements.

ABTS Decolorization assay

ABTS assay was performed following the procedures previously reported,[9] adapted to a methodology in microplate.[15] $ABTS^{\bullet+}$ solution (5 mM) was prepared by oxidizing ABTS with MnO_2 in distilled water for half an hour in the dark. Then, the solution was

filtered through filter paper. The absorbance of the $ABTS^{\bullet+}$ solution was then read at 734 nm in a spectrophotometer, adjusting solution absorbance to 0.700 by diluting with ethanol. To a 96-well microtitre plate assay were added 50 µL aliquot of different concentrations of extracts and 200µL of $ABTS^{\bullet+}$ solution. After incubation at room temperature for 10 min, the absorbance of each well was determined at 734 nm using a microplate reader (FLUOstar Optima, BMG Labtech microplate reader). The radical scavenging activity was expressed as the Trolox equivalent (TE) per g of extract.

DPPH assay

Free radical scavenging activity of the extracts on DPPH free radical was estimated according to the method of Srinivasan *et al.*,[16] adapted to a methodology in microplate. The DPPH assay was used as a rapid spectroscopic method to provide an evaluation of antioxidant activity due to scavenging free radicals. Being a stable free radical colored purple, DPPH free radical is reduced to the yellow diphenylpicryl hydrazine. Stock solutions of DPPH (100 µM) in ethanol used and the solution was covered from light before use. The same procedure as the ABTS experiment was applied, except for using the DPPH solution instead of the ABTS working solution. The absorbance was read at 517 nm. Assay of free radical scavenging activity was repeated three times for each sample and the means were reported. Trolox®, was tested in the assay as reference compound.

FRAP assay

The FRAP values of stock solutions of different extracts /compounds were determined according to previously published method of Firuzi *et al.*,[17] with some modifications.The FRAP reagent was prepared by mixing solution A (10 parts) [300 mM acetate buffer pH 3.6], with solution B (1 part) [2,4,6-Tris(2- pyridyl)-s-triazine (TPTZ) 10 mM dissolved in 40 mM HCl]. The FRAP reagent was warmed to 37 °C before used. Aliquots of extracts or components were added in triplicate onto wells of a 96- well plate (flat-bottomed, Nunc). The assay was started by adding 180 µL of FRAP reagent to each well. Appropriate blanks were prepared from the solvent and run simultaneously with the samples. The plate was immediately shaken in a FLUOstar Omega plate reader for 30 sec and the reaction was allowed to run for 10 min after which the plate was read on a plate reader (595 nm). A reference solution of Trolox was run simultaneously and used to generate the calibration curve by linear regression. The standard curve was linear between 25 and 800 mM Trolox (TE). Results were expressed in mM TE g^{-1} of extracts. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

Agar disk diffusion test

Antimicrobial activity of aqueous and ethanol extracts of honeysuckle berries was tested using the agar disc diffusion method. Bacterial and fungal strains included were *S. aureus* ATCC (American Type Culture Collection, Rockville, Md.) 25923, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212, *C. albicans* ATCC 24433. Clinical and Laboratory Standards Institute (CLSI) guidelines were followed to perform the antimicrobial disc susceptibility test.[18] A few colonies from an overnight culture of each bacterial strain were resuspended into saline and diluted appropriately to reach about 108 cells per mL. *C. albicans* was processed similarly but the density of the inoculum, which was prepared at 106 cells per mL. The prepared bacteria suspensions were spread onto Mueller Hinton agar plates (4 mm thick) using a sterile cotton swab. Solid RPMI medium (4 mm thick) was used for *C. albicans*. Sterile blank paper discs (6 mm in diameter, Oxoid, Basingstoke Hampshire, England) were placed on the surface of the inoculated media and spotted with 10 µL of the extract (20 mg mL^{-1}). Ciprofloxacin (5 µg disc) and Nystatin (100 Units disc) were used as reference antimicrobials against bacteria and fungi, respectively. Bacterial plates were incubated at 37 ± 1 °C for 24 h, while *C. albicans* was incubated for 48 h. Clear and transparent halos indicated antimicrobial activity. The inhibition zone diameter (IZD) was measured with a ruler and expressed in mm. The paper disc diameter was included in reported IZDs, therefore only a value above 6 mm meant activity.

Cytotoxicity on tumor cells

Quantification of cell viability is based on the reduction of the yellow dye thiazolyl blue tetrazolium bromide to a purple formazan product by living cells. The A375 human malignant melanoma cell line and the MDA-MB 231 human breast adenocarcinoma cell line were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 2 mM L-glutamine, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). All cells were grown in a humidified atmosphere at 37 °C in the presence of 5% CO_2. Constituents and supplements of the growth media were purchased from Sigma (St. Louis, MO, USA). Cell viability was determined by the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This assay was carried out following the protocol of Quassinti *et al.*[19] The cells were diluted to obtain a density of 2 x 104 cells mL^{-1}. 100 µL of this cell suspension were put into each well of a 96-well plate. After 24 h, samples were exposed to different concentrations of blue honeysuckle fruit (0.39–200 µg mL^{-1}). The vehiculum used, water and water/ethanol (1:1 v/v), were applied as a negative control, while cisplatin was used as positive control. Cells were incubated for 72 h. 10 µL of MTT (5 mg mL^{-1} in phosphate-buffered saline, PBS) was added to each well and plates were incubated at 37 °C for 4 h. The purple formazan product was dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance was recorded at 540 nm with a Titertek Multiscan microElisa spectrophotometer (Labsystems, Helsinki, Finland). Experiments were conducted in triplicate. Cytotoxicity was expressed as the concentration of extract showing a lethal effect on 50% of the cells (IC_{50}). To calculate the IC_{50} values the GraphPad Prism 4 computer program (GraphPad Software, 4. Diego, CA, USA) was used.

Proliferation assay

Human fibroblasts cells were cultured in Minimum Essential Medium containing 15% fetal bovine serum (FBS), 2mM L-glutamine, 100 U mL^{-1} penicillin and 100 µg mL^{-1} streptomycin and incubated at 5% CO_2 at 37° C. Blue honeysuckle fruit aqueous extract was dissolved in water at a concentration of 10 mg mL^{-1} and diluted with the cell culture medium prior to use. 7000 cells of human fibroblasts cell line were suspended in 98 µL of specific medium and incubated in a 96 well plate for overnight. After the incubation, 2 µL of the aqueous extract were added to the well with

the final concentrations of 50, 100, 150 and 200 μg mL^{-1}. After 12, 24, 48 and 72 h of incubation at 37° C, viability of the cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)- 2-(4-sulfenyl)-2H-tetrazolium (MTS) assay using Cell Titer 96Aqueous One Solution Cell Proliferation assay (Promega Italia S.R.L.).[20] After the addiction of MTS, in combination with the electron coupling agent phenazine methosulfate, the cells were allowed to incubate for 1 h and absorbance was measured at 492 nm in a microplate reader, GeniosPro. Cell viability was calculated as a percentage using the formula: (mean OD of treated cells/mean OD of control cells) x 100. Results are expressed as percent of control cells which are not treated. An untreated control, a positive control (10 nM epidermal growth factor, EGF) and a control with water were run. All experiments were done in triplicate.

Wound healing-migration assay

Human fibroblast cells were grown to confluence on a 6-well plate, the medium was aspirated and fresh 2% FBS-medium with or without several concentrations of *L. caerulea* aqueous extract was added. The cells were scratched once per well with a P10 pipette tip to create an artificial wound (0.5–1 mm) and the wound was allowed to heal for 24 hours. The average extent of wound closure was evaluated by measuring the area of the wound. The speed of migrating cells into the wound area was examined and photographed. The fibroblasts were treated with various concentrations of test formulation and were examined at 0, 12 and 24 h after treatment. Untreated control and positive control (10 nM EGF) were also maintained. Fibroblasts were treated with 50, 100, 150 and 200 μg mL^{-1} and incubated for 24 h. The extent of cell migration was photographed at the same location and measured using ImageJ as Image analyzing software. Each experiment was performed in triplicate.[21]

Immunomodulatory activity

Three healthy volunteers (male, age: 43 ± 4 years) were enrolled in the study. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood samples (10 mL/human) and the in vitro PBMC proliferation assay was performed by staining PBMC (2 $x10^5$/well live cells in flat bottom 96-well tissue culture plates) with carboxyfluorescein diacetate succinimydyl ester (CFSE; BioLegend, San Diego, CA) cell tracer.[22] To selectively activate the T cells, some wells of the culture plates were prepared the day before by an overnight coating with a goat anti-mouse IgG (H+L) (Southern Biotech) and then by using a mouse anti-human CD3 monoclonal antibody (anti-hum CD3 mAb; Clone UCTH1, BioLegend, San Diego, CA) for 2 h at 37°C before the seeding of the isolated PBMCs. The excess of the monoclonal antibody was aspirated among the last two steps. Further utilized proliferation stimuli were: 1 μg mL^{-1} of pokeweed mitogen (PWM, it stimulates the B lymphocyte only in the presence of T cells; Sigma-Aldrich) or 1.2 μg mL^{-1} of phytohemagglutinin (PHA, a polyclonal T-cell activator; BiochromAG, Berlin) in presence or absence of the Russian Berry extract (L) diluted in HBSS medium at the final concentration of 200 μg mL^{-1}. Each culture condition was repeated in triplicate. A negative control was represented by PBMC cultured without any mitogen/activator (CTR) and *L. caerulea* extract, so that the base proliferation could be estimated.[23] Flow cytometry analysis was performed after culture using a different combination of monoclonal antibodies: PE labeled mouse anti-human CD8 mAb (Clone HIT8a, BD Biosciences) and PECY5 labeled mouse anti-human CD4 mAb (Clone No. RPA T4; BD Biosciences), PE labeled mouse anti-human CD20 mAb (Clone 2H7; BD Biosciences) and APC labeled mouse anti-human CD56 mAb (Clone NCAM 16.2; BD Biosciences) or APC labeled mouse anti-human CD79a mAb (clone HM47, BD Pharmigen). For CD79a staining, cells were permeabilized with 0.1% saponin blocking buffer.[24] Samples were acquired on a standard FACSCaliburTM flow cytometer (Becton Dickinson, Mountain View, CA) operating the CELLQuestPro TM software. Data were analyzed by gating on proliferating lymphocytes (according to CFSE dilution). All the blood donors provided written informed consent in accordance with the declaration of Helsinki. The study was approved by the local Ethics Committee (CEAS Umbria). The results were analyzed using the GLM procedure of SPSS v.13. An ANOVA model, with the mitogens/activators (anti-hum CD3, PHA and PWM), monoclonal antibody (mAb) and treatment (CTR vs *L. caerulea*) as fixed variable, was used. Data are expressed as least squares means and standard error (SEM). Differences were considered to be significant when $P \leq 0.05$

Results and discussion

Quantification of polyphenols and chlorogenic acids

We performed the simultaneous analysis of eleven compounds, among polyphenols and caffeoylquinic acids, i.e. shikimic acid, gallic acid, (+)-catechin hydrate, (-)-epicatechin, p-coumaric acid, trans-resveratrol, caffeic acid, trans-ferulic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid, in the blue honeysuckle berries aqueous and ethanolic extracts (Table 1).

Table 1 Quantitative determination of the analyzed compounds in blue honeysuckle ethanolic and acqueous extracts samples (μg g^{-1} dry weight plant material); relative standard deviations were in a range from 0.78 to 6.88 % (n=3)

Compounds	Extract	
	Ethanolic	Acqueous
Shikimic acid	204.4	51.16
Gallic acid	n.d.	n.d.
(+)-Catechin hydrate	n.d.	n.d.
(−)-Epicatechin	n.d.	n.d.
p-Coumaric acid	n.d.	n.d.
trans-Resveratrol	n.d.	n.d.
5-O-Caffeoylquinic acid	67.89	29.01
3-O-Caffeoylquinic acid	741.4	233.1
3,5-di-O-Caffeoylquinic acid	n.d.	n.d.
Caffeic acid	n.d.	n.d.
trans-Ferulic acid	n.d.	n.d.
Total content	1013.69	313.27

n.d. means not detected

Only shikimic acid, 3-O-caffeoylquinic acid and 5-O-caffeoylquinic acid were present in the two extracts analyzed. 3-O-caffeoylquinic acid (chrlorogenic acid) was the most abundant compound in both extracts, followed by shikimic acid and 5-O-caffeoylquinic acid (neochlorogenic acid). These metabolites resulted more abundant in the ethanolic extracts, with concentrations of 741.4, 204.4 and 67.89 μg g^{-1}, respectively. To caffeoylquinic acid derivatives, such as

3-O-caffeoylquinic acid, and 5-O-caffeoylquinic acid, a lot of pharmacological properties can be ascribed, among which antioxidant, antibacterial, antihistaminic, hepatoprotectant and neuroprotective activities.[25] The occurrence and amount of these hydroxycinnamic acids in *L. caerulea* extracts is consistent with various studies that reported the following amounts: 304-1562 μg g^{-1} from berries cultivated in US,[26] and up to 760 μg g^{-1} from berries harvested in horticultural farms in Poland.[27,28] Furthermore, the values of these compounds found in honeysuckle are comparable to those of blueberries (1149 μg g^{-1}) and black currants (580-930 μg g^{-1}),[29] but lower with respect to bilberries (1130-2310 μg g^{-1}).[30] According to Palikova *et al*.[31] blue-berried honeysuckle may also contain epicatechin and phenolic acids such as ferulic, caffeic, and coumaric acids that instead were not detected in our samples.

Quali-quantitative determination of anthocyanins

In the present study, five individual anthocyanins, in particular diglucosides and glucoside of three anthocyanidins (cyanidin, peonidin, pelargonidin), were identified using HPLC/DAD-ESI-MS. In Table 2 the individual compounds analyzed, together with molecular weight, relative fragments obtained from mass analysis and amount of the detected antocyanins (mg g^{-1} dry weight) are reported. The most abundant anthocyanin in honeysuckle berry was cyanidin-3-glucoside (10.763 mg g^{-1}), followed by cyanidin-3,5-diglucoside (0.807 mg g^{-1}), peonidin-3-glucoside (0.666 mg g^{-1}) and pelargonidin-3-glucoside (0.081 mg g^{-1}). Cyanidin-3-glucoside represents 87% of the total anthocyanins amount (12.317 mg g^{-1}), cyanidin-3,5-diglucoside 7%, peonidin-3-glucoside 5% and pelargonidin-3-glucoside 1%. These relative percentages of antocyanins are in agreement with those reported in previous studies on honeysuckle berries.[26,32,33] On the other hand, the concentrations of antocyanins found in our sample coming from spontaneously growing plants are much higher with respect to those detected in some cultivars from Polish farms. In fact, *Oszmianski et al*.[27] found 2.95 mg g^{-1} of cyanidin-3-glucoside, 0.0739 mg g^{-1} of cyanidin-3,5-diglucoside, 0.0355 of mg g^{-1} peonidin-3-glucoside and 0.0076 mg g^{-1} of pelargonidin-3-glucoside. *Wojdyło et al*.[28] detected 2.22-8.05 mg g^{-1} of cyanidin-3-glucoside, 0.046-0.420 mg g^{-1} of cyanidin-3,5-diglucoside, 0.045-0.469 mg g^{-1} of peonidin-3-glucoside and 0.0092-0.0225 mg g^{-1} of pelargonidin-3-glucoside. Also when compared with wild growing plants from Poland,[34] the levels of cyanidin-3-glucoside in the Russian samples were significantly higher (10.763 vs 1.118-3.321 mg g^{-1}, respectively). The high amount of cyanidin-3-glucoside found in our samples is of particular interest as this compound occurs in several health-promoting fruits.[31]

Table 2 Identification and quantification of anthocyanins in blue honeysuckle berries (mg g^{-1} dry weight)

Compound	Molecular (m/z)	Fragments (m/z)	amount
Cyanidin-3,5- diglucoside	611	449, 287	0.807
Peonidin-3,5- diglucoside	625	463, 301	< LOD
Cyanidin-3-glucoside	449	287	10.763
Pelargonidin-3-glucoside	433	271	0.081
Peonidin- 3-glucoside	463	301	0.666
Sum of Anthocyanins			12.317

Proximate analysis and fatty acid composition

The results of the chemical composition and estimated energetic value obtained for blue honeysuckle fruit is shown in Table 3, where values of nutrients are reported on a fresh weight basis. The moisture content was 87.67%. Protein was found to be 2.12%, meanwhile carbohydrates was 0.86%. Level of fat was very low (0.01%). Crude fiber, an important component with beneficial effects to organism, was quite high (8.34%). The percentage of ash oscillated between proteins and fat content (0.45%). These values which signify the inorganic part of the fruits are eloquent of high mineral content.

Table 3 Proximate composition and mineral content of the honeysuckle berries. Values, referred to fresh matter, are means of three determinations ± SD.

	blue honeysuckle berries
Moisture	87.67±3.7
Macronutrients (%)	
Protein	2.12±0.2
Carbohydrate	0.86±0.1
Sugars	0.24±0.1
Fat	0.01±0.0
Ash	0.45±0.1
Crude fibre	8.34±0.1
Energy (Kcal)	50±2.0
Ascorbic acid (mg 100 g^{-1})	22.5±0.3
Minerals (mg kg^{-1})	
Ca	1030±22
Mg	1020±20
Na	863±14
K	324±8
Cu	124±6
Zn	203±7
Fe	128±6
Ag	traces

On the basis of the proximate analysis, it was calculated that one hundred g of blue honeysuckle berries provided about 50 kcal. The mineral contents of blue honeysuckle fruits are given in Table 3. Minerals play essential functions such as skeletal structure, maintenance of colloidal system and regulation of acid-base equilibrium; they also are components of hormones, enzymes and enzyme activators. The honeysuckle berries are rich in macro-elements such as Ca (1030 mg kg^{-1}), Mg (1020 mg kg^{-1}), Na (853 mg kg^{-1}) and K (324 mg kg^{-1}). In particular, the amount of Mg and Ca found in our samples were close to those reported by Pokorná-Juríková and Matuškovič,[35] who found an average level of 710 mg kg^{-1} for Mg and of 1077 mg kg^{-1} for Ca, but lower with respect to data by Lefevre *et al*.[36] who found level of 2660 mg $kg^{-1}$of Ca and of 1157 mg kg^{-1} of Mg. On the other side, the amount of Na in our sample was 10 times higher with respect to that found by Pokorná-Juríková and Matuškovič[35] (81 mg kg^{-1}) which instead detected very high level of K (12235 mg kg^{-1}). Also Lefevre *et al*.[36] found high level of K (17770 mg kg^{-1}), but they did not detect Na in honeysuckle fruits. As a consequence of the good nutritional value of honeysuckle berries, the levels of some macro-elements found in our study, such as Ca, Mg and Na, are particularly higher with respect to those found in blueberry (213, 82 and 1.4 mg kg^{-1} respectively) that instead contained K in high concentration (684 mg kg^{-1}).[37] Other authors did not detect Ca and Na in blueberry fruits, but they found K at high level (701 mg kg^{-1} against 324 mg kg^{-}

[1] found in our sample).[38] In our work, some interesting microelements were found in high amount such as Cu (124 mg kg^{-1}), Zn (203 mg kg^{-1}) and Fe (128 mg kg^{-1}). Interestingly also traces of Ag were detected. The levels of these minerals are much higher with those reported for honeysuckle berries by various authors[36-38] that found low level of Cu (0.4-12.4 mg kg^{-1}), Fe (1.3-29.1 mg kg^{-1}) and Zn (1-10.0 mg kg^{-1}).

The method developed for quantitative determination of ascorbic acid in honeysuckle berries showed a level of 22.5 mg $100g^{-1}$ (Table 3). The determinations were performed in triplicate with a RSD% value lower than 8.03%. This content is a bit lower with respect to those reported in berries from *L. kamtschatica* and *L. edulis* cultivated in an experimental area of Slovak Republic[35] in which levels of ascorbic acid ranged from 28.56 up to 86.96 mg $100g^{-1}$ fw.

Table 4 Relative fatty acids percentages in blue honeysuckle fruit

Fatty acid	%[a,b]
Hexadecanoic acid (palmitic acid)	5.3
Octadecanoic acid (stearic acid)	0.9
(*Z*)-9-Octadecenoic acid (oleic acid)	22.8
(*Z*)-11-Octadecenoic acid (*cis*-vaccenic acid)	1.3
(*Z*,*Z*)-9,12-Octadecadienoic acid (linoleic acid)	66.8
(*Z*,*Z*,*Z*)-9,12,15-Octadecatrienoic acid (linolenic acid)	2.9
SFAs[c]	6.1
MUFAs[d]	24.2
PUFAs[e]	69.8

[a] Weight percentages have been obtained from the FAMEs GC-FID analysis, after applying theoretical response factors to the area values obtained.[45] [b] Relative standard deviations % obtained (n = 3) were in the range 2.4-12.5%. [c] SFAs: saturated fatty acids. [d] MUFAs: mono unsaturated fatty acids; [e] PUFAs: polyunsaturated fatty acids.

Fatty acid (FA) composition of blue honeysuckle berries is mostly represented by unsaturated FAs (93.9 %, Table 4) with linoleic acid as the most abundant (66.8 %), followed by oleic acid (22.8 %), then by palmitic acid (5.3 %) and linolenic acid (2.9 %). Other FAs detected in lower amounts were cis-vaccenic acid and stearic acid. FA composition is much different from that found by Palikova *et al.*[31] for berries from the same variety harvested in Central Moravia (Czech Republic, i.e., Central Europe, out of its natural habitat) where the authors specify that palmitic and oleic acids dominated, while polyunsaturated FA were found in small amount. Taking into consideration the recommended high levels of unsaturated compared to saturated FAs in a daily diet, FAs composition of blue honeysuckle fruits can be considered having a high nutritional quality.

Antioxidant activity

Antioxidant activity of blue honeysuckle berries extracts is reported in Table 5. The berries displayed a total phenolic content of 18.6 and 32.7 mgGAE g^{-1} for ethanol and aqueous extract, respectively, that were higher than those reported for several cultivars of *L. caerulea* var. *kamtschatica* grown in Czech Republic,[39] but lower that those found in cultivars of the same species from China[32] and Russia.[36] We assume that these differences may depend on the different extraction method that was used by authors, in addition to genetic and geographic factors.

Table 5 Total phenolic content and antioxidant capacity of ethanol and aqueous extract of blue honeysuckle fruit

blue honeysuckle fruit	TOTAL PHENOLS mgGAE g^{-1}	DPPH TEAC[a] µmol TE g^{-1}	DPPH IC_{50}[b] µg mL^{-1}	ABTS TEAC[a] µmol TE g^{-1}	ABTS IC_{50}[b] µg mL^{-1}	FRAP TEAC µmol TE g^{-1}
Ethanol extract	18.6± 6.1	103± 4.5	212± 9.2	421± 37	4.2± 0.4	376± 26
Aqueous extract	32.7± 4.8	72.5± 7.7	332± 35	596± 1.3	14.3± 0.0	404± 4.1
Positive control						
Trolox			6.00± 0.5		3.50± 0.2	

[a]GAE: Gallic acid equivalent. [b]TEAC: Trolox equivalent (TE) antioxidant concentration; Values are referred to dry matter. [c] IC_{50}: The concentration giving a reduction of 50%.

Overall, the blue honeysuckle berries gave TEAC values that were in the ranges 103-421 and 72.5-596 µmol TE g^{-1} for ethanol and aqueous extract, respectively. The highest activity of extracts was shown on ABTS radical, with the ethanolic extract having a comparable activity to that of Trolox (IC_{50} of 4.2 µg mL^{-1}) and the aqueous one only four times lower (IC_{50} of 4.2 µg mL^{-1}). This results can be related to the higher content of anthocyanins and chlorogenic acids exhibited by ethanolic extracts (Tables 1-2). In DPPH assay both extracts exhibited a lower scavenger effect with IC_{50} values 35 and 55 times lower than that of positive control. Also in this case the radical scavenger power of the ethanolic extract was higher than that of aqueous extract (IC_{50} of 212 and 332 µg mL^{-1}, respectively).

Similar TEAC values were obtained for both extracts in the FRAP assay (376 and 404 µmol TE g^{-1} for ethanol and aqueous extract, respectively) corresponding to a noteworthy reducing capacity (Table 5). The obtained results appear consistent with those already reported in literature for blue honeysuckle berries of different geographic origin. In particular, blue-berried samples from Polish plantation showed comparable antioxidant activity with TEAC values in the ranges 170-417 and 93-166 µmol TE g^{-1} in ABTS and DPPH tests, respectively.[34] Instead, several honeysuckle varieties and genotypes cultivated in Poland showed comparable activity in ABTS assay (TEAC values in the range 12.65-49.73 mmol TE $100g^{-1}$), but lower activity in FRAP assay (TEAC values in the range 3.53-7.42 mmol TE $100g^{-1}$). Also in this case, the different method of extraction together with differences in genetics and geographic origin may contribute to the variation in activity observed.

Antimicrobial activity and cytotoxicity on tumor cells

Table 6 reports the inhibition zone diameters (IZDs) produced by *L. caerulea* polar extracts on a panel of pathogens (two gram-positive, two gram-negative and one yeast). The inhibition zone diameter produced by both fruit extracts on microorganisms was in the range of 6 to 7 mm that means no activity.

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Table 6 Antimicrobial activity of blue honeysuckle ethanol and aqueous extracts determined by the agar disk diffusion test. Values indicate the diameter of the growth inhibition zone (mm)

	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	*C. albicans*
Ethanol extract					
L. caerulea	6	6	6	6	7
Aqueous extract					
L. caerulea	7	6	7	6	6
Reference antibiotics					
Ciprofloxacin	28	23	30	31	n.r.
Nystatin	n.r.	n.r.	n.r.	n.r.	27-28

n.r.: Not recommended for this species.

From the MTT assay (data not shown) resulted that both *L. caerulea* fruit extracts were inactive as antiproliferative agents on A375 and MDA-MB 231 tumor cell lines showing IC_{50} values above 200 µg mL^{-1} that are far higher than 20 µg mL^{-1} that is considered the threshold to evauate a plant extract as a promising source of cytotoxic drugs by US NCI.[40]

Proliferation activity

The blue honeysuckle aqueous extract absorbance test, achieved with the MTS assay, was performed in order to detect its effect on the viability of human fibroblast cells. The analysis of the absorbance data, obtained using 50, 100, 150 and 200 µg mL^{-1} of aqueous extract at several time points, has underlined that the extract is inactive. In fact, it did not show toxic effects and did not influence the proliferation rate on human fibroblasts cell line also at 200 µg mL^{-1}. However, the positive control EGF showed 70% increase in cell growth, while cell proliferation was insignificant both in untreated control and in control with water.

Migratory activity

The blue honeysuckle aqueous extract slightly enhanced the cell migration of human fibroblasts when tested by scratched wound assay. In Fig. 1 it is possible to appreciate the migratory activity of these cells after 0, 12 and 24 h of incubation with I) the negative control, II) the 50, 100, 150, 200 µg mL^{-1} of *L. caerulea* extract and III) the positive control. The results showed that after 12 h of treatment with all concentrations of the extract the migratory nature is comparable to that of negative control. After 24 h of incubation with 50 and 100 µg mL^{-1} of extract, there was a negligible migratory activity. On the other hand, in the case of treatment with 150 and 200 µg mL^{-1} a slight

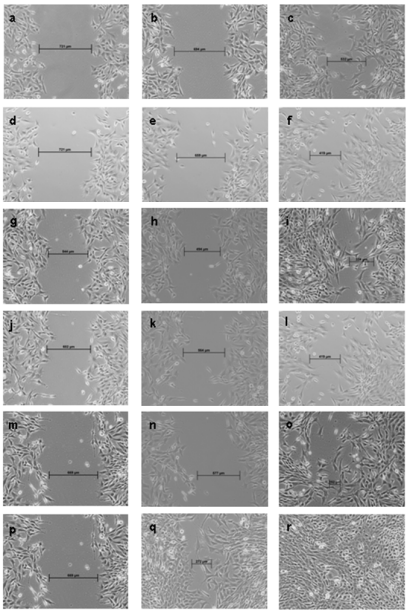

Fig. 1 Cell Migration Assay. a) Control-0 hours, width 100%; b) Control-12 hours, width 93%; c) Control-24 hours, width 51%. d) 50 µg mL^{-1}-0 hours, width 100%; e) 50 µg mL^{-1}-12 hours, width 95%; f) 50 µg mL^{-1}-24 hours, width 50%. g) 100 µg mL^{-1}-0 hours, width 100%; h) 100 µg mL^{-1}-12 hours, width 92%; i) 100 µg mL^{-1}-24 hours, width 45%. j) 150 µg mL^{-1}-0 hours, width 100%; k) 150 µg mL^{-1}-12 hours, width 91%; l) 150 µg mL^{-1}-24 hours, width 41%. m) 200 µg mL^{-1}-0 hours, width 100%; n) 200 µg mL^{-1}-12 hours, width 89%; o) 200 µg mL^{-1}-24 hours, width 40%. p) Positive control-0 hours, width 100%; q) Positive control-12 hours, width 54%; r) Positive control-24 hours, width 4%.

migratory nature of fibroblast was visible in comparison with negative control. More in detail, after 24 h we can detect a 41 and 40% of wound width (in comparison to the 100% at time 0) after treatment with 150 and 200 μg mL^{-1} of extract, respectively. Conversely, at the same time point, the blank has shown to close less than half of the wound, while the positive control tended to a complete closure of the scratch. Since the blue honeysuckle aqueous extract did not show toxic effects on fibroblasts and produces a slight effect in wound healing it could be interesting to thoroughly study the effects of these fruits in cytotoxic stress and DNA damage induced by UVA and UVB rays.[41]

Immunomodulatory activity

In Fig. 2 the PBMC proliferation percentages obtained in the in vitro culture with/without the honeysuckle aqueous extract and/or mitogens/activators are shown (CFSE dilution assay). As expected, the mitogens/activators (anti-hum CD3 mAb, PWM and PHA; positive controls) induced a consistent cell proliferation compared to CTR cells. Of interest, the honeysuckle extract seemed to exert an inhibitory effect on PBMC proliferation (CTR cells or cell cultured with PWM and anti-hum CD3, Fig. 2). At least, at the dosage of 200 μg mL^{-1}. Significant effects of the interaction between mitogen/activator * fruit extract (*P*<0.036), treatment (with or without *L. caerulea*, *P*<0.008) and mitogen/activator stimuli (*P*<0.001) were obtained. Only the cells cultured with *L. caerulea* and both the activators of T cells (anti-hum CD3 mAb and PHA) seemed to have minimized the "inhibitory" effect of the honeysuckle extract, showing a pattern similar to that one obtained by cells cultured without the fruit aqueous extract, but in the same conditions.

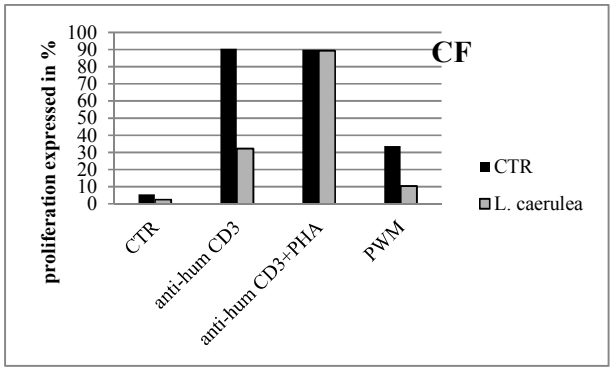

Fig. 2 Human peripheral blood mononuclear cells (PBMC) proliferation assay monitored by CFSE labeling in response to in vitro stimulation with *Lonicera caerulea* aqueous extract. Data are shown as means (standard error: 9.89) and it was observed a significant effect of interaction between mitogen/activator * fruit extract (*P*<0.036).

The T lymphocytes require two independent signals to become fully activated. The first, an antigen-specific signal, is sent via the unique antigen receptor (i.e. antibodies to the CD3/Tcell receptor complex). The second signal, namely the costimulation, is independent of the antigen receptor and is critical to allow full activation, sustain cell proliferation, prevent anergy and/or apoptosis.[42] In our study, the costimulation required to obtain the full activation of the T cells seems to have been inhibited by *L. caerulea* when there was not a simultaneously activation from PHA (its exclusive combination with Russian fruit was not investigated in the present study). It is reasonable to speculate that *L. caerulea* may hamper the full activation of T cells by counteracting or competing with some costimulatory signals or/and by inducing apoptosis. PHA, a lectin found in plants, acts as polyclonal activator of T cell proliferation that probably uses different tolerance pathways from those utilized by the anti-hum CD3 mAb and costimulatory molecules present in the culture, eluding the inhibitory effect of the honeysuckle berries extract on the proliferating cells. The finding of a possible inhibitory effect of honeysuckle berries on activation/proliferation of immune cells, such as its possible role in promoting cell apoptosis need to be confirmed in a higher number of cases.

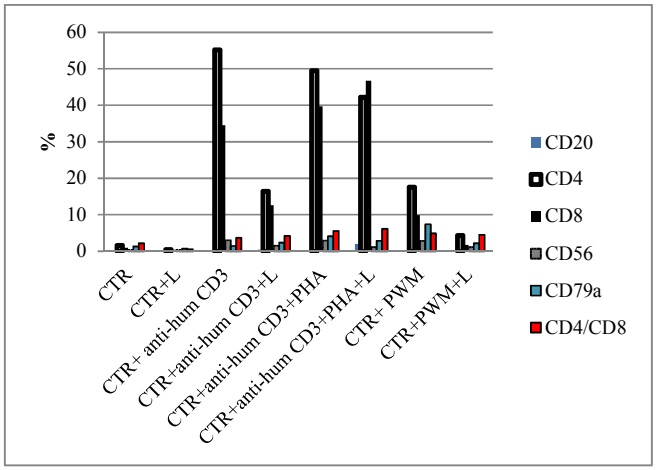

Fig. 3 Lymphocyte subset variations (expressed in percentages) obtained after five day of in vitro stimulation or not (CTR) with *Lonicera caerulea* aqueous extract (L) and/or mitogen/activator (PHA, PWM and anti-hum CD3). Data are shown as means and significant treatment (L) effects were observed for CTR, anti-hum CD3 and PWM cultured cells (*P*<0.03, 0.001 and 0.000; respectively).

The interplay of the various costimulatory and inhibitory signals are expression of the high complexity in the cell-cell interactions regulating the lymphocyte activation. Several aspects regarding lymphocyte differentiation, effector cell regulation, memory cell generation and maintenance are still unknown.[43] The assessment of how plant extracts could interfere with lymphocyte activation, possibly by studying the interactions at a 'cell receptor level', may contribute in the comprehension of these complex mechanisms underlying lymphocytic activation.

In Fig. 3 the different lymphocyte subsets obtained in the proliferated cell stimulated or not with mitogen/activators and/or honeysuckle extract for five days are represented. Consistent treatment effects induced by *L. caerulea* extract were observed in the proliferated cells cultured without any stimuli (CTR) or cultured with anti-hum CD3 mAb and PWM (0.03, 0.001 and 0.000, respectively). Conversely, significant interactions between treatment and mAb were obtained for cells cultured with anti-hum CD3 mAb (*P*<0.011) and PWM (*P*<0.001).

Interestingly, the PBMC cultured with both anti-hum CD3 mAb + PHA and *L. caerulea* showed an inversion of the CD4/CD8 ratio (CD8+ higher than CD4+) vs those obtained in absence of honeysuckle extract. It seems that when a T cell immune response is stimulated, the presence of the honeysuckle aqueous extract in the culture could induce a shift towards a cytotoxic (CD8+ cells) immune response that could represents the mechanism by which anthocyanins exert their well documented, although elsewhere, marked ability to reduce cancer cell proliferation and to inhibit tumor formation.[44]

Conclusion

The potentiality of blue honeysuckle berries to give bioactive compounds used for food and nutraceutical applications has recently attracted attention of scientists. Our study confirmed the richness in phenolic compounds, mainly anthocyanins, of these berries supporting their use in food industry, as preservative, or in dietary supplements supporting health conditions where oxidative processes are involved. At the same time our analysis showed that spontaneous plants contain comparable amounts of these health-promoting molecules with respected to cultivars previously examined. Moreover, this study approved the good nutritional value of honey suckle berries due to their high content in healthy macro- and micro-nutrients. Our investigation showed the lack of activity of these fruits neither as antibiotics nor as antiproliferative agents towards tumor cells. Instead, only a slight fibroblasts migratory activity potentially useful to enhance the effect of a wound healing drug was observed. The honeysuckle extract could be a valid candidate in sun protective and after-sun dermatological preparations or more in general, in cosmeceutic preparations although more studies are needed to validate this application. Finally, the blue honeysuckle berries seem to exert an immune modulatory activity which differs depending on whether concern the B or T lymphocytes. However, this effect needs to be confirmed and further investigated in a higher number of cases in order to clarify a potential risk related to the consumption of these berries and their derivatives.

Acknowledgements

This work was supported by the FAR 2014-2015 (Fondo di Ateneo per la Ricerca) of the University of Camerino. Authors thank Dr. Evy Blomme from Gent University for her precious support in the chemical and biological analyses.

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Author contribution statement

As corresponding author for the manuscript entitled 'Blue honeysuckle fruit (*Lonicera caerulea* L.) from eastern Russia: phenolic composition, nutritional value and biological activities of its polar extracts'

I DECLARE THAT

all co-authors have agreed to this submission for publication and accepts the responsibility for having properly included all (and only) co-authors.
Please below the contribution of each co-author is reported

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Yours Sincerely,
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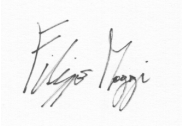

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