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Bergamot (*Citrus bergamia* Risso) Fruit Extracts as γ-Globin Gene Expression Inducers: Phytochemical and Functional Perspectives

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Epicarps of *Citrus bergamia* fruits from organic farming were extracted with the objective of obtaining derived products differently rich in coumarins and psoralens. The extracts were chemically characterized by ^{1}H nuclear magnetic resonance (NMR), gas chromatography−flame ionization detection (GC-FID), gas chromatography−mass spectrometry (GC-MS), and high-pressure liquid chromatography (HPLC) for detecting and quantifying the main constituents. Both bergamot extracts and chemical standards corresponding to the main constituents detected were then assayed for their capacity to increase erythroid differentiation of K562 cells and expression of γ-globin genes in human erythroid precursor cells. Three experimental cell systems were employed: (a) the human leukemic K562 cell line, (b) K562 cell clones stably transfected with a pCCL construct carrying green-enhanced green fluorescence protein (EGFP) under the γ-globin gene promoter, and (c) the two-phase liquid culture of human erythroid progenitors isolated from healthy donors. The results suggest that citropten and bergapten are powerful inducers of differentiation and γ-globin gene expression in human erythroid cells. These data could have practical relevance, because pharmacologically mediated regulation of human γ-globin gene expression, with the consequent induction of fetal hemoglobin, is considered to be a potential therapeutic approach in hematological disorders, including β-thalassemia and sickle cell anemia.

KEYWORDS: Bergamot extracts; nonvolatile fraction; psoralens; coumarins; NMR; GC-FID; GC-MS; HPLC; erythroid differentiation; K562 cells; fetal hemoglobin; β-thalassaemia

INTRODUCTION

Bergamot is a small and roughly pear-shaped citrus fruit, growing on small trees known as bergamots (*Citrus bergamia* Risso, Rutaceae). Bergamot plants, despite their uncertain species definition and phytogeographical origin, are mainly cultivated in Ionian coastal regions of southern Italy, Argentina, and Brazil for their fruits. In fact, bergamot fruits are traditionally employed as raw material in cosmetics and cosmeceutics because of their essential oil flavor and in dietary and herbal products as crude drugs because of the eupeptic properties and therapeutic activities against digestive disorders (*1*).

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Bergamot oil is extracted by cold pressing of peels, and it is made up of 93−96% volatile compounds, such as monoterpenes, in particular 25−53% limonene, and high quantities of oxygenated compounds, such as linalool (2−20%) and linalyl acetate (15−40%) (*2*). A variable percentage of the essential oil (4−7%) consists of nonvolatile compounds as pigments, waxes, and above all coumarins (e.g., citropten) and psoralens (e.g., bergapten and bergamottin) (**Figure 1**). The quality and quantity of the nonvolatile fraction represent important parameters in terms of efficacy and safety for use of bergamot essential oil and derived products for health. While bergamot coumarins and psoralens show interesting bioactivities with therapeutic possibilities with, for example, anti-inflammatory, antianginal, and antiarrhytmic properties (*3*−*5*), the same chemicals have long been known to induce allergenic effects and severe skin diseases due to their photoreactivity (*6*, *7*).

Fang et al. (*8*) demonstrated that the vacuum fractionated distillation performed on bergamot oil obtained by cold pressing led to the deterpenation and partial removal of oxygenated

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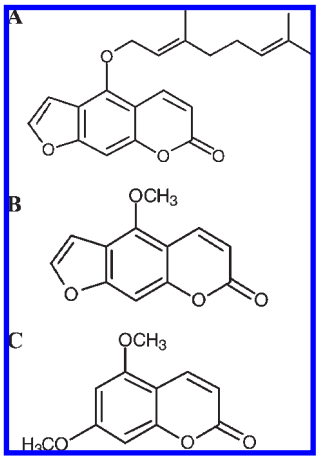

Figure 1. Molecular structures of bergamottin (5-geranyloxypsoralen, **A**); bergapten (5-methoxypsoralen, **B**); and citropten (5,7-dimethoxycoumarin, **C**).

compounds (providing enriched oil with nonvolatile and oxygenated compounds), whereas extraction with supercritical fluids was more appropriate for the separation of oxygenated compounds from nonvolatile fractions. Starting from this chemical and biological evidence, epicarps from mature commercial bergamot fruits organically grown were processed through extraction strategies to obtain three different kinds of extracts gradually richer in psoralens and coumarins.

The aim of this study was to determine whether these different bergamot extracts are able to induce erythroid differentiation of human erythroid cells and to identify and quantify through GC-FID, GC-MS, HPLC, and ^{1}H NMR which compound, or compounds in a synergic combination, could be responsible for the bioactivity.

The identification and characterization of potential therapeutic agents in hematological diseases, including β-thalassaemia and sickle cell anemia, are based on several approaches, including pharmacologically mediated regulation of the expression of human γ-globin genes (*9*−*13*). Therefore, several recently published experiments were designed to find hormones, cytotoxic agents, hemopoietic cytokines, and short fatty acids as agents able to increase HbF levels in humans (*10*−*13*). Growing experimental evidence clearly indicates that (a) not all of the patients are responders to single HbF inducers and (b) the combined treatment with different HbF inducers is much more effective than the treatment with single compounds. This paper reports a strategy to identify novel products from nature able to induce HbF in primary human erythroid cells.

To determine the biological activity of the different bergamot extracts and the pure compounds detected and identified, three different experimental cell systems were employed: (a) human leukemic K562 cell line (*14*, *15*), (b) K562 cell clones stably transfected with a pCCL construct carrying the green fluorescence protein under the transcriptional control of the γ-globin gene promoter, and (c) the two-phase liquid culture of human erythroid progenitors isolated from normal donors (*16*). These experimental approaches were useful in identifying molecules capable of stimulating HbF production in erythroid precursors derived from normal subjects as well as patients with thalassemia and sickle cell anemia (*17*).

MATERIALS AND METHODS

Plant Material and Extraction Procedures. Commercial mature *C. bergamia* fruits belonging to three different stocks from organic farming in southern Italy were purchased and manually processed to completely remove the epicarp. The raw plant material obtained (150 g for each sample stock) was immediately suspended in 600 mL of chloroform and processed for the extractions. The suspension was homogenized for 5 min and submitted to sonication in an ultrasound bath (Ultrasonik model 104X, Ney Dental Inc.) in the dark at a constant temperature of 25 °C. Subsequently, the samples were filtered and centrifuged for 20 min at 3000 rpm. The residue was re-extracted with 400 mL of chloroform following the same procedure previously described. The collected chloroform extracts, named Extract 1, were partially reduced in volume with a Rotavapor and then completely dried under nitrogen flow. The total extraction yield was 3.6 ± 0.2%. Aliquots (5 g) of these sample extracts (Extract 1) were subjected to fractional distillation at 350−400 Pa and 40 °C, as described elsewhere (*7*), to completely remove volatile terpene compounds and partly oxygenated chemicals. The sediment obtained at the end of the distillation corresponded to the Extract 2 samples with an extraction yield of 40.3 ± 0.8%. One gram of samples of Extract 2 was then resuspended in 20 mL of diethyl ether, vigorously shaken for 30 min, and centrifuged. The supernatant was removed, and the residue, corresponding to Extract 3 samples, was collected, dried under nitrogen flow, and weighed (yield = 65.1 ± 1.5%). For all laboratory processing care was taken to protect the operations from light and oxidizing conditions. All of the solvents used were purchased from Sigma-Aldrich and were of Reagent European Pharmacopoeia purity. All of the samples (Extract 1−3) were stored in the dark in glass vials with Teflon-sealed caps at 2.0 ± 0.5 °C until analyses by GC-FID, GC-MS, HPLC, and ^{1}H NMR to identify and quantify putative bioactive compounds.

^{1}H NMR Fingerprinting. ^{1}H spectra were recorded on a Varian Gemini-400 spectrometer operating at 399.97 MHz and at temperature of 303 K. Sample extracts (10 mg) were dissolved in deuterated chloroform (0.8 mL) in a 5 mm NMR tube; solvent signal was used for spectra calibration (^{1}H 7.26 ppm). ^{1}H spectra were run using a standard pulse sequence "s2pul". ^{1}H NMR spectra in $CDCl_3$ of bergapten, citropten, linalool, and limonene [Spectral Database for Organic Compounds, SDBS—free site organized by National Institute of Advanced Industrial Science and Technology (AIST), Japan, at http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/cre_index.cgi?lang=eng], linalyl acetate (Sigma-Aldrich NMR spectra database), and bergamottin (*7*) are completely described in the literature.

GC-FID and GC-MS Analyses. The sample extracts were analyzed and the relative peak areas for volatile and oxygenated components weighted by GC-FID. The relative percentages were determined using GC TRACE Thermoquest, equipped with autosampler Triplus from the Thermo Electron Corp. The column was a VF-5 ms, 30 m × 0.25 mm. Flow rate was 1.0 mL/min He, and the split was 1:50. Ohter conditions: injector temperature, 300 °C; detector temperature, 350 °C; oven temperature, initially 55 °C, raised to 100 °C at a rate of 1 °C/min, and raised to 250 °C at a rate of 5 °C/min, and finally held at that temperature for 15 min. One microliter of each sample (15 mg/mL CH_2Cl_2) was injected. The percentage composition of volatile and oxygenated components was computed by the normalization method from the GC peak areas (data integration software: Jasco-Borwin version 1.5, JMBS Developments, Fontaine, France), without correction factors. Identification was performed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer with electron impact and hooked to a NIST library. A Varian FactorFour VF-5 ms poly-5% phenyl-95%-dimethylsiloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm) was used. Operating conditions were as follows: injector temperature, 300 °C; FID temperature, 300 °C; carrier (helium) flow rate, 1.0 mL/min; and split ratio, 1:50. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 μA; scan rate, 1 scan/s; mass range, 29−400 Da; trap temperature, 150 °C; transfer line temperature, 300 °C. The main compounds were identified by comparing their relative retention time, KI, and MS fragmentation pattern with those of essential oils of known composition, with pure compounds, and by matching the MS fragmentation patterns with the above-mentioned mass spectra libraries and with those in the literature (*18*). To determine the Kovats index of the components, an alkenes mixture (C_8−C_{24}) was added to the sample extracts before injection in the GC-MS equipment and analyzed under the same conditions as above.

HPLC Analysis. HPLC analysis was performed to quantify the main constituents of the nonvolatile fraction of bergamot essential oil. Therefore, pure commercial standards of bergaptol, bergamottin, bergapten,

citropten, and 5-geranyloxy-7-methoxycoumarin (Extrasynthese, Genay, France) were used as external standards to set up and calculate appropriate calibration curves. The experimental conditions were performed using a Jasco modular HPLC (Tokyo, Japan, model PU 2089) coupled to a diode array apparatus (MD 2010 Plus) linked to an injection valve with a 20 μL sampler loop. The column used was a Tracer Extrasil ODS2 25 × 0.46 cm, with a flow rate of 1.0 mL/min. The mobile phase employed consisted of solvent solution B (methanol) and A (water/formic acid = 95:5). The gradient system adopted was characterized by four steps: 1, isocratic, with solvent solution B/A = 40:60 (%), for 2 min; 2, solvent solution B raised progressively from 40 to 60% in 18 min (from min 2 to min 20) until reaching the ratio B/A = 60:40 (%); 3, solvent solution B then raised to 100% in 4 min (from min 20 to min 24); 4, solvent solution ratio reached B/A = 40:60 in 4 min (from min 24 to min 28). Injection volume was 40.0 μL. Chromatograms were recorded, and peaks from bergamot sample extracts were identified by comparing their spectra with spectra obtained with pure standards. Peak area was determined by integration using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France). The qualitative and quantitative analysis of each extract was performed three times.

Cell Lines and Culture Conditions. Human leukemia K562 cells (*19*) were cultured in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Analitical de Mori, Milan, Italy), 50 units/mL penicillin, and 50 mg/mL streptomycin (*20*). Cell growth was studied by determining the cell number per milliliter with a ZF Coulter Counter (Coulter Electronics, Hialeah, FL). Treatment with bergamot extracts and chemical inducers was carried out by adding the appropriate drug concentrations at the beginning of the experiment (cells were usually seeded at 30000 cells/mL). The medium was not changed during the induction period. Erythroid differentiation was determined by counting benzidine positive cells after suspending the cells in a solution containing 0.2% benzidine in 0.5 M glacial acetic acid and 10% H_2O_2, as elsewhere described (*20*).

K562 Cell Clones Transfected with a pCCL Construct Carrying Green-EGFP under the γ-Globin Gene Promoter. High-throughput approaches are needed for screening of HbF inducers. Recombinant clones for the screening of high numbers of potential HbF inducers were designed and produced, preferentially acting on the human γ-globin gene promoter pCCL.promβ.HcRed1.promγ.EGFP. This vector was employed, carrying the enhanced green fluorescence protein (EFP) gene under the control of γ-globin gene promoter (*21*). Human K562 cells were used to obtain stable transfectants. In this system, an increase of green EGFP signal is consistent with a γ-globin gene promoter driven activity. Cells were seeded at 12500 cells/mL and treated with the appropriate concentration of chemical inducers to determine the activity of the compounds in inducing the expression of γ-globin genes. After 5 days of culture, cells were assayed for fluorescent protein expression. First, they were analyzed under a fluorescence inverted microscope, using filters suitable for green FPs. The fluorescence intensity was then determined by using a Wallac 1420 Victor^3 Multilabel Counter (Perkin-Elmer, Waltham, MA). Cells were harvested, washed, and counted. The same amounts of cells were then lysed with PBS 0.1% Triton and centrifuged to remove cell debris. Finally, 100 μL of cell lysate was analyzed using a Victor^3 Multilabel Counter. The detection of green fluorescence was carried out in OptiPlate 96 (Perkin-Elmer, Waltham, MA), by using a 485 nm excitation filter and a 590 nm emission filter; the lamp energy was 3000.

Human Erythroid Cell Cultures from Normal Donors. The two-phase liquid culture procedure was employed as previously described (*22*, *23*). Mononuclear cells were isolated from peripheral blood samples of normal donors by Ficoll−Hypaque density gradient centrifugation and seeded in α-minimal essential medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% FBS (Analitica de Mori, Milano, Italy), 1 μg/mL cyclosporin A (Sigma-Aldrich), 10% conditioned medium from the 5637 bladder carcinoma cell line (*24*), and 10 ng/mL stem cell factor (SCF, PeproTech EC Ltd., London, U.K.). The cultures were incubated at 37 °C, under an atmosphere of 5% CO_2 in air, with extra humidity. After 7 days of incubation in this phase I culture, the nonadherent cells were harvested, washed, and recultured in fresh medium composed of α-medium, 30% FBS, 1% deionized bovine serum albumin (Sigma-Aldrich), 10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, 10^{-6} M dexamethasone, 1 U/mL human recombinant erythropoietin (Tebu-bio, Magenta, MI, Italy), and 10 ng/mL SCF. This part of the culture is referred to as phase II (*22*). Bergamot extracts were added on days 4−5 of phase II, and cells were harvested on day 12 of phase II.

Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction (PCR). RNA was isolated and reverse transcribed as elsewhere described (*25*). Quantitative real-time PCR assay (*25*, *26*) of γ-globin mRNA and α-globin transcripts was carried out using gene-specific double-fluorescent labeled probes in an ABI Prism 7700 Sequence Detection System version 1.7.3 (Applied Biosystems, Monza, Italy). The following primer and probe sequences were used: γ-globin forward primer, 5′-TGG CAA GAA GGT GCT GAC TTC-3′; γ-globin reverse primer, 5′-TCA CTC AGC TGG GCA AAG G-3′; γ-globin probe, 5′-FAM-TGG GAG ATG CCA TAA AGC ACC TGG-TAMRA-3′; α-globin forward primer, 5′-CAC GCG CAC AAG CTT CG-3′; α-globin reverse primer, 5′-AGG GTC ACC AGC AGG CAG T-3′; α-globin probe, 5′-FAM-TGG ACC CGG TCA ACT TCA AGC TCC T-TAMRA-3′; β-globin forward primer, 5′-CAA GAA AGT GCT CGG TGC CT-3′; β-globin reverse primer, 5′-GCA AAG GTG CCC TTG AGG T-3′; β-globin probe, 5′-FAM-TAG TGA TGG CCT GGC TCA CCT GGA C-TAMRA-3′. The fluorescent reporter and the quencher were FAM and TAMRA, respectively. For real-time PCR the reference gene was 18S. This probe was fluorescent-labeled with VIC (Applied Biosystems, Monza, Italy).

Analysis of Hb Production. The proportion of HbF (percentage of total Hb) was determined by HPLC (*22*, *23*), using a Beckman Coulter instrument System Gold 126 Solvent Module-166 Detector. The wavelength utilized to detect hemoglobins was 415 nm, eluting the samples in a solvent gradient using aqueous sodium acetate−BisTris−KCN buffers. The column utilized to separate the hemoglobins was a Syncropak CCM 103/25. The standard controls were the purified HbA (Sigma-Aldrich, Milano, Italy) and HbF (Alpha Wassermann, Bologna, Italy) (*27*).

RESULTS

Phytochemical Investigation. The phytochemical investigation of bergamot (*C. bergamia* Risso, Rutaceae) epicarp fruits was performed with two main aims: (i) to set up an extraction strategy that could give extracts with different qualitative and quantitative profiles of volatile and nonvolatile compounds and (ii) to set up a combined analytical system that could lead to a qualitative and quantitative determination of the chemical constituents of the extracts, with particular reference to nonvolatile fraction chemicals, that are coumarins and psoralens.

The link between these phytochemical aims and the bioactivity assays targets was to determine the role of the nonvolatile fraction, or of their constituents individually, with or without the synergic interaction of volatile chemicals, in the modulation of γ-globin gene expression in different in vitro cell models.

For the chloroform extract (Extract 1 samples), composition was similar to that in bergamot essential oil with two significant fractions: (a) monoterpenes and their correlated compounds and (b) coumarins and psoralens (*28*). The extraction procedures followed led to products (Extract 2 and Extract 3 samples) with a progressive decrease of volatile compounds and an increase of the nonvolatile fraction (coumarins and psoralens) as determined by semiquantitative combined GC-FID and GC-MS analyses (**Table 1**). Extract 1 samples showed linalyl acetate (36.12%), linalool (27.35%), and limonene (18.78%) as the main constituents, as for bergamot essential oil, whereas coumarins and psoralens made up 4.64% (**Table 1**). Extract 2 samples showed an important reduction of linalyl acetate (27.45%) and a dramatic decrease of other terpene compounds. Many minority chemicals were not detectable, whereas others, such as limonene, showed a reduction of 93.2%. Extract 3 samples gave an even more noticeable reduction of terpene compounds, with linalyl acetate (0.43%), γ-terpinene (0.17%), and β-myrcene (0.53%) as the only volatile fraction chemicals, against an increase of coumarins and

Table 1. Composition of Bergamot Extracts Determined by GC-MS and GC-FID

		area %[b]		
identified compound	KI[a]	Extract 1	Extract 2	Extract 3
α-pinene	939	0.56 ± 0.04	nd	nd
sabinene	976	0.60 ± 0.05	nd	nd
β-pinene	979	0.73 ± 0.05	nd	nd
β-myrcene	991	2.53 ± 0.21	2.51 ± 0.18	0.53 ± 0.06
α-terpinene	1017	0.10 ± 0.01	nd	nd
p-cymene	1025	0.13 ± 0.02	nd	nd
limonene	1027	18.78 ± 1.17	1.27 ± 0.11	nd
trans-*E*-ocimene	1050	0.15 ± 0.02	nd	nd
γ-terpinene	1060	6.37 ± 0.35	1.06 ± 0.12	0.17 ± 0.02
linalool	1097	27.35 ± 1.89	3.62 ± 0.23	nd
α-terpineol	1189	0.13 ± 0.01	nd	nd
nerol	1230	0.22 ± 0.02	0.56 ± 0.07	nd
linalyl acetate	1257	36.12 ± 2.48	27.45 ± 1.88	0.43 ± 0.04
geranial	1267	0.28 ± 0.02	0.89 ± 0.06	nd
γ-terpinyl acetate	1349	0.11 ± 0.03	1.75 ± 0.11	nd
neryl acetate	1362	0.31 ± 0.03	1.57 ± 0.11	nd.
geranyl acetate	1381	0.19 ± 0.02	1.42 ± 0.13	nd
caryophyllene	1409	0.37 ± 0.04	0.70 ± 0.08	nd
trans-α-bergamotene	1435	0.38 ± 0.03	1.03 ± 0.09	nd
β-bisabolene	1506	0.58 ± 0.07	1.23 ± 0.11	nd
coumarins, psoralens[c]		4.64 ± 0.44	52.60 ± 2.56	96.54 ± 2.34

[a] KI, Kovats indices calculated with Varian Factor Four VF-5 ms. [b] Calculated with GC-FID; nd, not detectable. [c] Only citropten and bergapten were detectable on GC-MS.

psoralens of 45.5 and 95.2% with respect to Extract 2 and 3 samples, respectively (**Table 1**).

HPLC analyses were performed to detect and quantify coumarins and psoralens reputed to be representative of the nonvolatile fraction and of the bioactivity of bergamot crude drug and derived products (*3*−*6*, *28*). This showed a homogeneous qualitative profile with respect to the occurrence of citropten, bergamottin, bergapten, bergaptol, and 5-geranyloxy-7-methoxycoumarin in all bergamot extract samples. A total amount corresponding to 5.83% was found in Extract 1 samples, 37.84% in Extract 2 samples, and 89.36% in Extract 3 samples (**Table 2**). Bergaptol and 5-geranyloxy-7-methoxycoumarin were not detectable in the three extracts, whereas the other compounds (citropten, bergamottin, bergapten) were always identified. The ratio among the detected chemicals changed greatly in the different kinds of samples. In samples of Extract 1 bergamottin and bergapten represented 2.57 and 2.89% respectively, whereas citropten was detected in lower concentrations (0.37%). In samples of Extract 2 concentrations of bergamottin, bergapten, and citropten were 9.44, 27.26, and 1.14%, whereas in samples of Extract 3 concentrations were 3.10, 85.75, and 0.51%, respectively (**Table 2**).

^{1}H NMR (**Figure 2**) was performed on all of the samples belonging to the three different extracts in order to have a fingerprint of the bergamot phytocomplexes. NMR can potentially perform in a single-step analysis the assessment of all organic compounds that constitute a phytocomplex (*29* and references cited therein). For the three extracts examined it was

Table 2. Coumarins and Psoralens Percentage (w/w ± SD) in the Bergamot Extracts Determined by HPLC Analyses

extract	citropten	bergamottin	bergapten	bergaptol	5-geranyloxy-7-methoxycoumarin
1	0.37 ± 0.03	2.57 ± 0.08	2.89 ± 0.07	nd[a]	nd
2	1.14 ± 0.07	9.44 ± 0.17	27.26 ± 0.59	nd	nd
3	0.51 ± 0.03	3.10 ± 0.06	85.75 ± 1.18	nd	nd

[a] nd, not detectable.

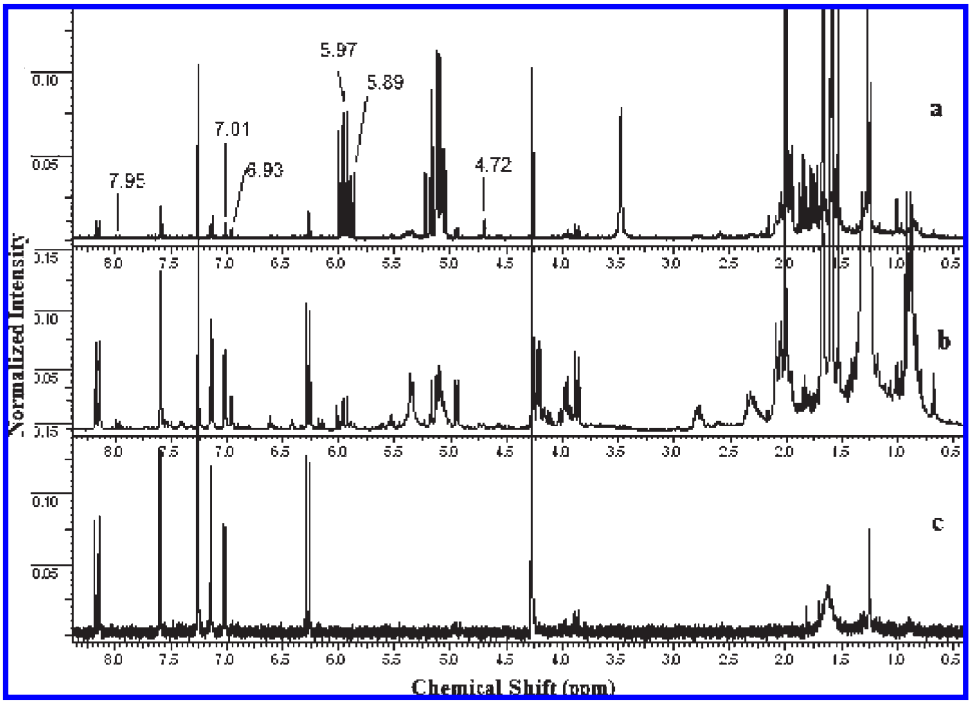

Figure 2. ^{1}H NMR spectra of samples belonging to Extract 1 (**a**), Extract 2 (**b**), and Extract 3 (**c**).

possible to detect, through a single NMR analysis, the presence of psoralens, coumarins, and monoterpenes.

This NMR approach allows with good approximation the relative amount of each detectable component by comparison of the integrals of typical not overlapped signals assigned to these chemicals to be obtained (*29*, *30*). In this specific case we considered two allyl protons on limonene (4.72 ppm), double doublet of proton on C_2 on linalool (5.89 ppm) and linalyl acetate (5.97 ppm), doublet of proton on C_3 on citropten (7.95 ppm), and doublet of proton near the oxygen on the furanic ring of bergapten (7.01 ppm) and bergamottin (6.93 ppm).

Growth and Differentiation of K562 Cells Cultured in the Presence of Bergamot Extracts. Figure 3A shows the effects of the bergamot extracts on the in vitro proliferation of K562 cells seeded in the absence or presence of different concentrations of the three preparations of bergamot extracts.

Figure 3B shows the effects of components of bergamot extracts (bergapten, bergamottin, and citropten) on K562 cell growth. The results indicate that all analyzed extracts caused a dose-dependent inhibition of cell proliferation. The IC_{50} values obtained from three indipendent experiments are shown in **Figure 4**, confirming the data of **Figure 3** and indentifying bergamottin as the compound exhibiting the highest antiproliferative activity ($p < 0.01$ compared to bergapten and citropten).

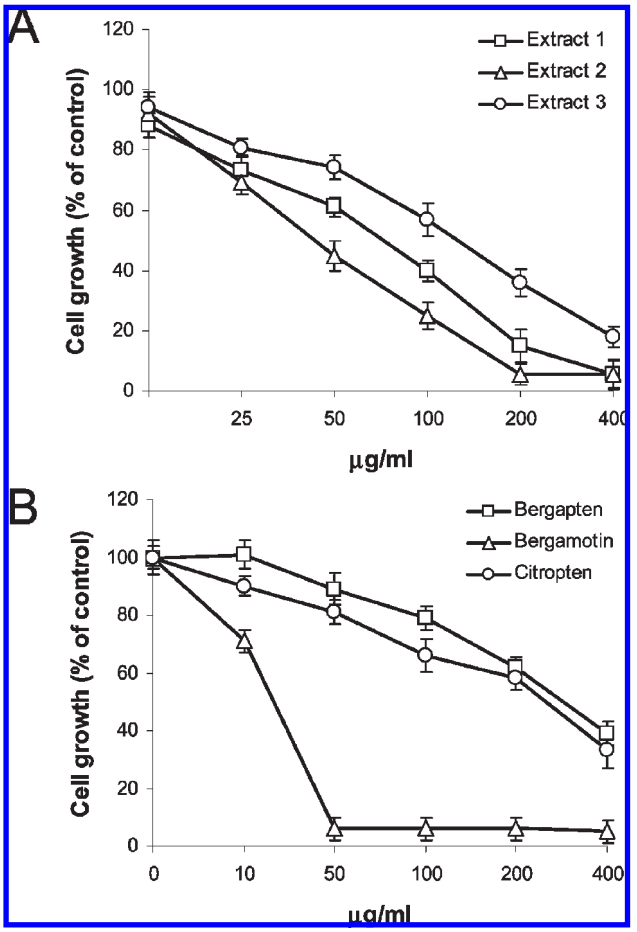

Figure 3. Effects of bergamot extracts and identified compounds on cell growth of K562 cells. Cells were cultured for 4 days in the presence of different concentrations (25−400 μg/mL) of Extract 1 (□), Extract 2 (△), or Extract 3 (○) (**A**) or in the presence of different concentrations (10−400 μM) of bergapten (□), bergamottin (△), or citropten (○) (**B**). The values of cell number per milliliter in treated cells were compared with untreated control cultures (taken as 100%). The results are the average ± SD for four different determinations of a single representative experiment.

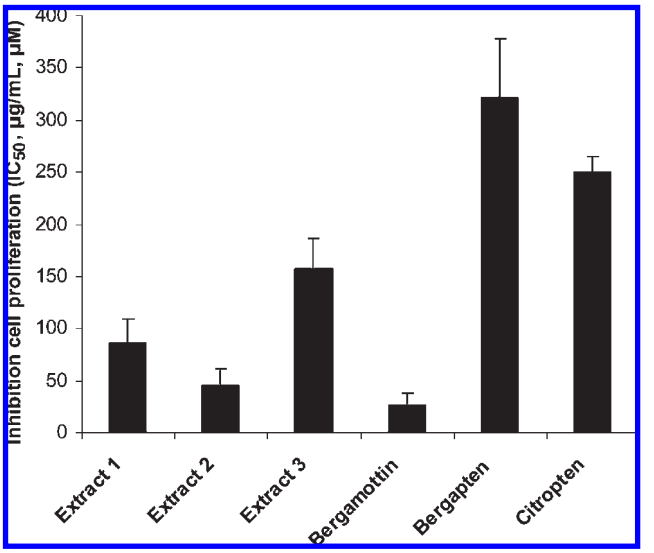

Figure 4. Inhibition of cell proliferation (IC_{50}, μg/mL or μM) of bergamot extracts and identified compounds on cell growth of K562 cells. The IC_{50} values were obtained from three independent experiments ± SD.

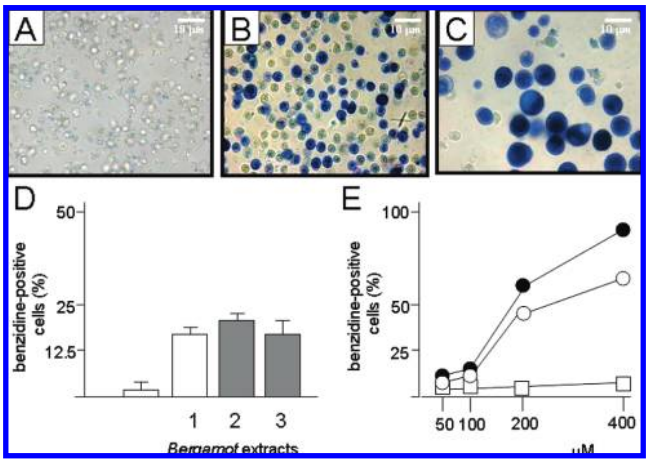

Figure 5. Effects of bergamot extracts and identified compounds on erythroid differentiation of human erythroleukemic K562 cells: (**A**−**C**) representative benzidine staining of untreated K562 cells (**A**) or K562 cells induced by 200 μM citropten (**B**) and 1 μM ara-C (**C**); (**D**, **E**) effects of 100 μg/mL bergamot extracts and increasing concentration of bergapten (○), citropten (●), or bergamottin (□) on K562 erythroid differentiation. K562 cells were treated with 100 μg/mL of bergamot extracts and identified compounds and the proportion of benzidine-positive (hemoglobin containing) cells was determined after 6 days.

The rationale of analyzing the biological effects of coumarins and psoralens was related to the fact that this class of compounds is the only one extensively represented in all of the bergamot extracts (**Table 1**).

The effect of bergamot extracts and pure compounds on K562 cell differentiation was determined by measuring the proportion of Hb-containing (benzidine positive) cells (**Figure 5**). Representative examples of erythroid-induced K562 cells are indicated in panels **A**−**C** of **Figure 5**. **Figure 5D** indicates that all three bergamot extracts display the ability to sustain K562 differentiation along the erythroid pattern. **Figure 5E** clearly indicates that the erythroid induction properties of bergamot extracts are due to the presence of bergapten (5-methoxypsoralen) and citropten (5,7-dimethoxycoumarin), whereas bergamottin (5-geranyloxypsoralen) was unable to sustain K562 erythroid differentiation. **Table 3** shows the level of induction of K562 cells by bergamot

Table 3. Effects of Bergamot Extracts and Identified Compounds on Erythroid Differentiation of Human Erythroleukemic K562 Cells Compared to Differentiation Agents Reported in the Literature (*29*, *30*)[a]

inducer	eythroid differentiation	concentration
Extract 1	23.1 ± 7.5	100 μg/mL
Extract 2	20.2 ± 5.4	100 μg/mL
Extract 3	25.4 ± 6.2	100 μg/mL
bergamottin	2.1 ± 0.8	400 μM
bergapten	60.5 ± 5.2	400 μM
citropten	70.1 ± 8.1	400 μM
Ara-C	78.3 ± 4.1	1 μM
mithramycin	85.6 ± 7.2	50 μM
butyric acid	35.3 ± 3.7	2.4 mM
hydroxyurea	28.2 ± 3.7	100 μM
cisplatin	62.5 ± 7.8	6 μM
angelicin	60.6 ± 6.2	400 μM
rapamycin	68.6 ± 10.2	100 μM

[a] The proportion of benzidine-positive (hemoglobin containing) cells was determined after 6 days of cell culture and obtained from three independent experiments ± SD.

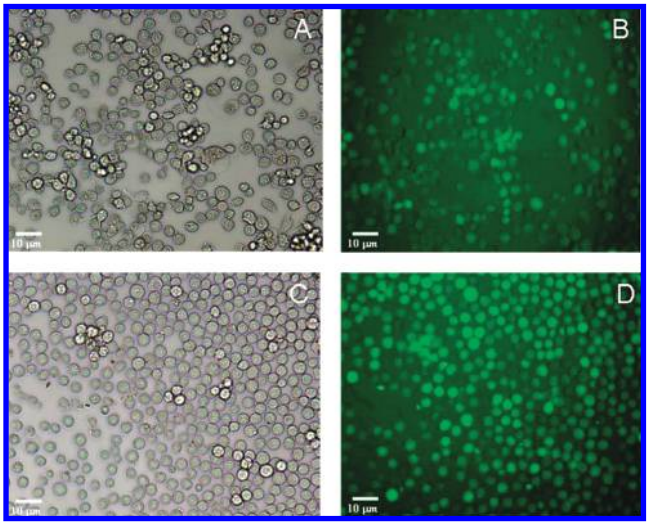

Figure 6. Effects of Extract 1 on γ-globin promoter driven transcription. A stable clone of K562 was obtained after transfection with the pCCL.promβ.HcRed1.promγ.EGFP and then cultured in the absence (**A**, **B**) or in the presence of 20 μg/mL Extract 1 (**C**, **D**). Analysis was performed after 5 days of incubation. Quantification of cell lysates was performed using the Wallac 1420 Victor[3] Multilabel Counter (Perkin-Elmer).

extracts and pure compounds in comparison with those of other known K562 erythroid inducers, such as cytosine arabinoside (ara-C), mithramycin, cisplatin, angelicin, butyric acid, and hydroxyurea (*25*, *26*). The data clearly show that bergamot extracts were low inducers of erythroid differentiation. Their level of induction was within the range of that displayed by hydroxyurea, the most frequently used molecules for experimental HbF inducing therapy of β-thalassaemia and sickle cell anemia (*17*). The efficiency of induction of erythroid differentiation shared by bergapten and citropten was similar to the level found with the best known inducers of K562 differentiation, such as mithramycin and ara-C. We suggest that the decrease of cell growth rate in treated K562 cells (**Figures 3** and **4**) is due to the activation of the terminal cell division of erythroid-induced cells, as the majority of treated cells (>95%) were negative to the trypan blue exclusion test (data not shown).

Given that both bergapten and citropten are able to induce erythroid differentiation, the next experiment on the bergamot extract focused on displaying the most similar amounts of bergapten and citropten (Extract 1).

Effects of Bergamot Extracts on K562 Cell Clones Stably Transfected with a pCCL Construct Carrying Green-EGFP under the γ-Globin Gene Promoter. To determine the effects of bergamot extracts, bergapten, and citropten on the transcription of γ-globin genes, a K562 cell clone stably transfected with the pCCL.promβ.HcRed1.promγ.EGFP construct was employed, carrying the green and red fluorescence protein genes, under the control of the γ-globin and β-globin promoters, respectively. This clone was cultured for 5 days in the presence or absence of bergamot extracts, bergapten and citropten. After this incubation period, cells were harvested and analyzed under a fluorescence inverted microscope. In addition, lysates were prepared and samples containing the same amounts of proteins analyzed using the Wallac 1420 Victor[3] Multilabel Counter (Perkin-Elmer). **Figure 6D** shows the results obtained indicating that Extract 1 induced an increase of green-EGFP positive with respect to untreated cells (**Figure 6B**), suggesting an inducing effect of the bergamot Extract 1 on γ-globin gene promoter activity. The data were confirmed and extended by FACS analysis. Results obtained show bergamot Extract 1 (**Figure 7A**), bergapten (**Figure 7B**), and citropten (**Figure 7C**) induce an increase of green-EGFP positive cells. Cotreatment with bergapten and citropten induced a further increase in the EGPF signal (**Figure 7D**). Panel **E** of **Figure 7** shows the quantitative data obtained in three independent experiments. These data were

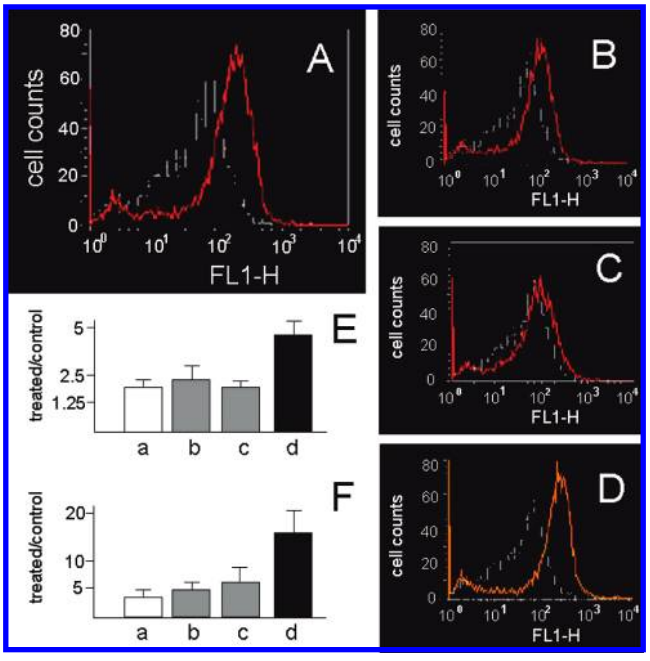

Figure 7. Representative effects of bergamot Extract 1 (**A**), bergapten (**B**), citropten (**C**), and bergapten plus citropten (**D**) on the expression of the green fluorescence protein gene under the control of the γ-globin gene promoter. A stable clone of K562, obtained after transfection with the pCCL.promβ.HcRed1.promγ.EGFP, was employed and cultured with Extract 1 (20 μg/mL), bergapten (300 μM), citropten (200 μM), and citropten plus bergapten (200 and 300 μM, respectively). In panels **E** and **F**, the data are relative to cells treated with bergamot Extract 1 (a), bergapten (b), citropten (c), and bergapten plus citropten (d). (**E**) Quantitative data of three independent experiments (data represent the average fold induction with respect to control untreated cells ± SD). Analysis was performed after 5 days of incubation. (**F**) Quantification of cell lysates using the Wallac 1420 Victor[3] Multilabel Counter (Perkin-Elmer) was performed at 485 nm for detection of the fluorescence activity carried by GFP.

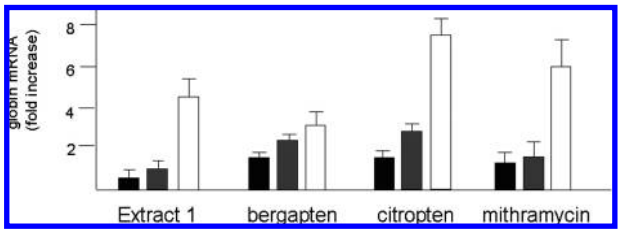

Figure 8. Effects of Extract 1, bergapten, and citropten on globin mRNAs of cultured erythroid progenitors: fold increase of α-globin (black columns), β-globin (dark gray columns), and γ-globin (white columns) mRNAs in erythroid progenitor cells treated with Extract 1 (100 μg/mL), bergapten (300 μM), citropten (200 μM), and mithramycin (25 nM). The results of untreated cells were taken as 1. Results represent the average ± SD of three independent experiments.

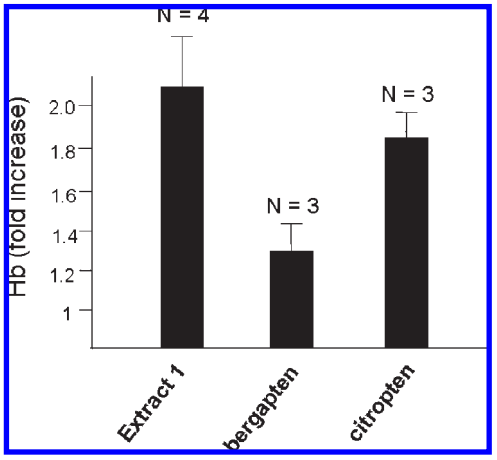

Figure 9. Effects of Extract 1, bergapten, and citropten on HbF production: HbF production by human erythroid precursor cells derived from peripheral blood and cultured in the absence or in the presence of Extract 1 (100 μg/mL), bergapten (300 μM), citropten (200 μM). Data represent percent of HbF (average ± SD of different experiments, $N = 3-4$, as indicated).

further confirmed by analysis of the fluorescence intensity using a Wallac 1420 Victor^3 Multilabel Counter. The same amounts of cells were lysed, and cell lysate was analyzed using a Victor^3 Multilabel Counter. **Figure 7F** shows the results obtained indicating that bergamot extracts, bergapten, and citropten induce an increase of fluorescence. Cotreatment with bergapten and citropten induced a further additive increase in the EGPF signal (black column of **Figure 7F**).

Accumulation of γ-Globin mRNA and HbF in Normal Human Erythroid Precursors. Early erythroid committed progenitors (BFUe) derived from the peripheral blood were isolated and allowed to proliferate and differentiate during phase I (in the absence of erythropoietin, EPO) into late progenitors (CFUe). They were treated in phase II with EPO to allow their proliferation and maturation into Hb containing orthochromatic normoblasts (*13*). Bergamot extracts, bergapten, and citropten were added on days 4−5 of phase II (when cells started to synthesize Hb). Cultures treated with mitramycin, a well-known potent inducer of HbF increase (*22*), were used as a positive control. RT-PCR analysis was performed to verify whether these treatments led to a preferential increase of γ-globin mRNA production in normal human erythroid precursors grown in the two-phase liquid culture system (*16*). 18S was employed as a reference gene. **Figure 8** shows that a clear increase of γ-globin mRNA occurs in bergamot-treated erythroid precursor cells. Increase of β-globin mRNA was also observed, although with some variability. This increase was always lower than that for γ-globin mRNA. No or very low increase of α-globin mRNA was detectable.

HPLC analyses of the cellular Hb content of these cultures showed that while the proportion of HbF in control cultures was 0.55 ± 0.6%, it increased to 2.31 ± 0.4% in bergamot extract-treated cultures (average ± SD of four experiments) (**Figure 9**). Also, bergapten and citropten were able to increase HbF production.

DISCUSSION

The identification of bioactive compounds within extracts from medicinal plants is a promising strategy for the development of possible pharmaceutically relevant molecules from nature. In the present paper, the phytochemical profile of bergamot epicarp extracts has been drawn with the objective of obtaining characterized fractions with increasing quantities of coumarins and psoralens. The ultimate goal was to identify single bioactive components able to induce erythroid differentiation of K562 cells and expression of γ-globin genes in human erythroid precursor cells. Therefore, a simple, rapid, and efficient extraction protocol to obtain both extracts chemically characterized as similar to bergamot essential oil (such as Extract 1 samples, which contain 95% terpenes) (*28*) and extracts progressively richer in coumarins and psoralens, but reduced in terpene compounds abundance, has been set up. As NMR can be performed to rapidly detect different classes of organic chemicals (*29* and references cited therein), it has been adopted as a rapid analytical research protocol for bergamot extracts. This allowed detection in a single analysis of the presence of coumarins, psoralens, and monoterpenes, collecting the same qualitative evidence for the main components obtained by GC-FID and HPLC. Quantitative results with good approximation comparing integrals of typical signals assigned to these chemicals were achieved (*29*, *30*). The phytochemical investigation was completed with the qualitative and quantitative determination of all terpene compounds and of coumarins and psoralens by the combined use of GC-FID, GC-MS, HPLC, and ^{1}H NMR.

Consequently, we stated that bergamot extracts are able to induce erythroid differentiation of K562 cells and, more importantly, increased expression of γ-globin genes and accumulation of HbF in human early erythroid committed progenitors (BFUe) derived from the peripheral blood. Among the compounds found in the bergamot extracts, bergamottin (5-geranyloxypsoralen) was inactive, whereas both bergapten (5-methoxypsoralen) and citropten (5,7-dimethoxycoumarin), when added on days 4−5 of phase, were able to induce a sharp increase of γ-globin mRNA without major changes in α-globin mRNA production. This is important for possible application in the therapy of β-thalassemia, because the β-thalassemic cells exhibit a large excess of α-globin. Accordingly, a good HbF inducer should not have any effects on the expression of α-globin genes. The RT-PCR analyses (**Figure 8**) demonstrated an increase, even if low, of β-globin mRNA, despite some variability. This increase was always lower than that of γ-globin mRNA. HPLC analyses demonstrated a significant increase of HbF in cultures treated with bergamot oil, bergapten, and citropten.

Therefore, the extracts and molecules presented in the present paper are of interest for analyzing their effects on cells from β-thalassemia patients. HbF production is a generally accepted approach to ameliorate clinical hematological parameters of β-thalassemic patients. It is indeed firmly established that inducers of HbF (such as hydroxyurea) might be able to convert β-thalassemia patients (requiring a regular transfusion regimen) to transfusion-independent subjects. Novel HbF inducers are

needed as some patients are refractory to the treatment and others develop resistance. HbF induction is clearly an important strategy in developing countries, where it is difficult to extend blood transfusion to all of the population due to the unavailability of blood and the risk of infection.

These results clearly indicate bergapten and citropten for possible development as HbF inducers. Further experiments are required to determine the safety, potential toxicity, in vivo bioavailability, and stability and whether suitable concentrations can be reached following in vivo administration.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; GC-FID, gas chromatography−flame ionization detection; GC-MS, gas chromatography−mass spectrometry; HPLC, high-pressure liquid chromatography; EGFP, enhanced green fluorescence protein; FP, fluorescence protein; MS, mass spectrometry; Hb, hemoglobin; HbF, fetal hemoglobin; RT-PCR, reverse transcription−Polymerase Chain Reaction; EPO, erythropoietin; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine; FBS, fetal bovine serum; PBS, phosphate buffer saline; NIST, National Institute of Standards and Technology; KI, Kovats index.

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