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Bioactive and antioxidant activity from *Citrus Bergamia* RISSO (BERGAMOT) juice collected in different areas of Reggio Calabria province, Italy

Vincenzo Sicari,[a]* Monica R. Loizzo,[b] Valentino Branca[a] and Teresa M. Pellicanò[a]

[a]Department of Agraria, University *"Mediterranea"* of Reggio Calabria, Località Feo di Vito, 89122 Reggio Calabria (RC) – Italy

[b]Department of Pharmacy, Health Science and Nutrition, University of Calabria, Via P. Bucci, Edificio Polifunzionale, 87036 Rende (CS) – Italy

***Corresponding author:** Vincenzo Sicari, Tel.: +39 0965324077; fax: +39 0965324077. E-mail address: vincenzo.sicari@unirc.it

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Abstract

The chemical composition and antioxidant activity of juice extracted from seven samples of bergamot (Citrus bergamia Risso) collected in different areas of Reggio Calabria Province were investigated. The ascorbic acid, total polyphenol, and flavonoid contents were determined. Total flavonoids and polyphenols were analyzed by UV spectra, while flavanone content was analyzed by HPLC. The antioxidant activity of the fractions was assessed using three representative assays: ABTS˙, DPPH˙ radical quenching and □-Carotene bleaching test. The main flavanones were naringin, neohesperedin and neoeriocitrin, and their average content 242.4±1.8, 183.0±0.6 and 247.0±1.4 mg mL-1 respectively. The results showed that bergamot juice possessed a good quality and a valuable source of health promoting constituents. In fact it contained eriocitrin, naringin, neoeriocitrin, and neohesperedin, which may contribute differentially to the antioxidant capacity.

INTRODUCTION

Bergamot (*Citrus bergamia* Risso) is thought to be a hybrid of the sour orange and citron or lemon. The fruit is spherical like an orange but yellow like a lemon. It has been known in the Mediterranean for several centuries and was described as early as 1708. Production is mostly limited to the Ionian coastal areas of the province of Reggio di Calabria (Italy). Bergamot is a symbol of the entire province, which grows about 90% of world production of this citrus fruit. This fruit is also cultivated in Côte d'Ivoire. However the quality of the obtained essence is not comparable due to the argillite, limestone and alluvial deposits found there.[1]There are three bergamot cultivars: Fantastico, Castagnaro and Femminello. Fantastico cultivar represents the most representative (90%).[2]

Citrus derivatives are an important field of food technology. However, even today, the technological processes often provide only partial extraction of all possible derivatives. As regards, in particular, bergamot, the aim of the producer is limited almost exclusively to the extraction of the essential oil; the juice and peel are used only occasionally, although they could be a source of profit for growers.

The juice is generally considered a waste product, which represents a serious environmental and economic problem for the industries. Most studies have focused on its essential oil as this constitutes a raw material for the perfume and food industries.[3-4] However, the presence of neoriesperidin, naringin, and neoeriocitrin in its juice has also been reported.[5-7]

Bergamot juice and albedo are rich in neoeriocitrin, neohesperidin, naringin, rutin, neodesmin and rhoifolin. The profile of the flavonoids in fruits of bergamot differs from other Citrus not only in terms of quality but also quantity.[8,9] Flavonoids are specially known for their antioxidant activities,[10- 12] which play a significant role in cardiovascular health and in prevention of cancer.[13-15] Other than fighting free radicals, they are also known for their antihistamine, antimicrobial, memory enhancing and even mood-boosting properties. In recent years, the beneficial properties of bergamot juice have been generating interest, and have been the subject of several studies.[16-20] Some researchers have focused on the molecular mechanisms of the bioactive compounds contained in the bergamot juice. The obtained results suggest that the flavonoids of bergamot juice may be useful for the development of alternative pharmacological strategies aimed at reducing the inflammatory process. [21-23]

Several studies have shown that flavonoids present in bergamot juice, lower cholesterol levels by modulating hepatic HMG-CoA levels.[24-26] Recently a research group of University of Catanzaro (Italy) has shown that administration of bergamot juice leads to a significant reduction in serum cholesterol and triglycerides. Moreover, the flavonoids present in the juice of this citrus fruit have a lipid-lowering action. Its effectiveness was tested on patients with hypercholesterolemia with and without type 2 diabetes, and on rats who also had an altered lipid profile. Intake of bergamot juice twenty minutes before meals helped reduce both cholesterol and triglycerides in patients. Moreover, none of the participants in the trial showed intolerance or side effects after taking the bergamot juice.[27] An analytical differentiation of Citrus juices, based on the differing concentrations of certain minor components, including flavonoids, plays an important role in determining chemotaxonomic markers to ascertain the authenticity of

commercial products.[28] The aim of the present paper was to evaluate the chemical composition and antioxidant activity in juice obtained from bergamot fruits collected Catona and Africo in a 90 km coastal stretch of the province of Reggio Calabria, Southern Italy.

MATERIALS AND METHODS

Plant Material and Juice Extraction

Citrus bergamia Risso fruits cv Fantastico were collected in February 2014 from plantations located between Catona and Africo (Reggio Calabria, Italy). Plantations were chosen in seven areas of the coast were bergamot is cultivated: Catona, Gallico, Arangea, Pellaro, Africo, Melito Porto Salvo, Palizzi (Fig. 1). All plantations were made up of plants aged 15-20 years and irrigated by sprinkler. Fruits were harvested at maturity stage and at full fruit size from a random sample of 15 plants in order to obtain a set of fruits that are representative. Plants received similar water and fertilizer treatments. Fruits were examined for integrity and absence of dust and insect contamination. Immediately after harvesting, the juice was extracted by mechanical pressure and frozen at – 80°C until analyzed.

Reagent

Hesperidin, narirutin, naringin, neohesperidin, neoeriocitrin, linoleic acid and β-carotene were purchased from Sigma Chemical Company (Milan, Italy). Ascorbic acid, Folin-Ciocalteu reagent and 2,2-diphenil-1-picrylhydrazyl radical (DPPH) and 2,2′-azinobis (3-ethyl-benzothiazoline-6-

sulphonate) (ABTS) were supplied by Carlo Erba (Milan, Italy). Solvents and reagents not expressly specified had a high degree of purity and were supplied by Carlo Erba (Milan, Italy).

Physicochemical analysis

The bergamot were squeezed and the juice was centrifuged and filtered to determine the following analyses: color of fresh juice was measured at 25°C using a Konica Minolta CM-700/600d spectrophotometer (Konica Minolta Sensing, Japan). Data were expressed as L* (lightness/darkness in a range 0-100), a* (greenness/redness in a range between -60 and + 60) and b* (blueness/yellowness in a range between – 60 and + 60). Total soluble solids (TSS) were determined using a digital refractometer PR-201α (Atago, Tokyo, Japan), previously calibrated at 20°C and the results expressed as degrees Brix; The pH was measured at ambient temperature with a pH meter (Model Basic 20, Crison) previously calibrated with standard solutions pH 4 and pH 7; Total acidity (TA) was determined using the International Federation of Fruit Juice producers test[29]: a potentiometric titration of the acidity of the juice, with a solution of 0.25 N NaOH up to pH 8.1. The results were expressed as g L^{-1} of anhydrous and hydrate citric acid. Ascorbic acid was determined using the International Federation of Fruit Juice producers test:[29] a potentiometric titration of the acidity of the juice, with a solution of 2,6-dichloroindophenol. Total flavanones were determined with a colorimetric method using alkaline diethylene glycol for determination of the bitter rhamnoglycoside naringin and other flavanones that may be present in citrus fruits.[30] The bergamot juice was analyzed for total phenolics by the Folin-Ciocalteu (FC) colorimetric method. [31]

All determinations above described were made in triplicate.

Flavanone analysis

Flavanone glycosides, expressed as hesperidin equivalents (mg L^{-1}), were determined by liquid chromatograph according to the official methodologies of the International Federation of Fruit Juice producers.[29] Fresh bergamot juice previously centrifuged at 4000 rpm for 20 minutes, was filtered through a 0.45 μm membrane filter. Separation of flavanones was performed by HPLC using a Phenomenex C18 column (150mm x 3 mm). The solvent system was buffer solution A: ACN/H_2O/H_2PO_4 (70:26:4) and buffer solution B: KH_2PO_4 pH 3.5, with flow-rate 500 μL/min in gradient conditions. The analysis was monitored at 287 nm. The gradient program was as follows: starting condition, 85% A, 15% B; 5 min, 70% A, 30% B; 20 min, 50% A, 50% B; 30 min, 25% A, 75% B; 35 min, 5% A, 95% B; 40 min, 85% A, 15% B. The column was operated at 25°C and flow rate was 1 mL min^{-1}. Identification of compounds was performed by comparing their retention time with those of standards and confirmed with characteristic spectra using the photodiode array detector.

Flavanone glycoside evaluation was calculated according to the external standard method by integration of the peak areas of individual compounds to that of standard curve prepared using hesperidin standard.

Antioxidant activity

DPPH assay: This experimental procedure was described by Loizzo et al. [32] In an ethanol solution of DPPH radical (final concentration was 1.0 x 10^{-4} M), samples at different concentrations were added. The reaction mixtures were shaken and kept in the dark for 30 min.

The absorbance of the resulting solutions was measured in 1 cm cuvettes using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer at λ= 517 nm against blank without DPPH. A decrease of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as % DPPH radical-scavenging that is calculated in the equation:

% DPPH radical-scavenging = [1 − (sample absorbance with DPPH-sample absorbance without DPPH/control absorbance)] × 100

ABTS assay: ABTS assay was based on the method previously described by Loizzo et al. [32] with slight modifications. ABTS radical cation ($ABTS^{+}$) was produced by the reaction of a 7 mM ABTS solution with 2.45 mM potassium persulphate. The mixture was stored in the dark at room temperature for 12 h before use. The $ABTS^{+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at λ= 734 nm. After addition of 25 µL of sample or Trolox standard to 2 mL of diluted $ABTS^{+}$ solution, absorbance was measured at exactly 6 min after mixing. Appropriate solvent blanks were run in each assay. The scavenging ability of the sample was calculated according to the following equation: ABTS scavenging activity (%) = $[(A_0 - A) / A_0] \times 100$ where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

β-Carotene Bleaching Test: Antioxidant activity was determined using β-carotene bleaching as previously described by Menichini et al. [33] Briefly, β-carotene solution was added to linoleic acid and 100% Tween 20. The emulsion was mixed with of samples at different concentrations and tubes were placed at 45°C in a water bath for 60 min. Propyl gallate was used as standard.

The absorbance of the samples, standard and control was measured at 470 nm against a blank at t = 0 and successively at 30 and 60 min.

Statistical analysis

All experiments were carried out in triplicate. Data were expressed as means ± standard deviation (S.D.). Analysis of variance (one-way ANOVA) was conducted using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL) and the Tukey's test was used to determine any significant difference among all treatments at $P < 0.05$. The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The dose-response curve was obtained by plotting the percentage inhibition versus concentration. Differences within and between groups were evaluated by one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test compared with the positive controls.

RESULTS AND DISCUSSION

The bergamot juice extracted from fruits collected in seven different areas of the coast of the province of Reggio Calabria (Italy), were examined in order to determine their antioxidant activity as related to their phenolic composition. Table 1 shows the areas where the fruits were collected with their latitude and longitude. There were significant differences ($P < 0.05$) in all color attributes among samples studied (Table 2). With respect to lightness (L*), the lowest value

corresponded to the Melito, Catona and Palizzi samples, while the highest values were those samples from Gallico, Arangea and Pellaro.

Table 3 shows the values of the chemical-physical characteristics (pH, titratable acidity, soluble solid and formol index) of the bergamot juice. The pH values of the juices analyzed are within normal range (2.54-2.84) and the differences between them are significant ($P < 0.05$). The lowest value was in the Africo sample and the highest (pH 2.84) in the Catona sample. Titratable acidity was significantly different: values were in the range 11.38-14.83. Soluble solid content measured for bergamot juice was in the range 8.11-11.61 °Brix. The formol index was used to estimate the total content of amino acids in a juice, and partly to estimate its purity. In the seven juices analyzed the values obtained show that there are significant differences.

Table 4 shows that the ascorbic acid, total flavonoid and total polyphenol content in all treated samples was significantly different ($P < 0.05$). Vitamin C (ascorbic acid), flavonoid and phenolic compounds are essential components for human health and have a high antioxidant activity, providing protection against free radicals and consequently participating in the prevention of many degenerative diseases. Ascorbic acid content was in the range 89.40-285.35 mg 100 mL^{-1}. The Gallico sample was significantly lower (89.4±2.1 mg 100 mL^{-1}) than that of other samples. Similarly, the total flavonoid content showed the lowest value for the Gallico and Palizzi samples (51.1±2.2 mg 100 mL^{-1} and 74.0±2.1 mg 100 mL^{-1}, respectively). The highest content of total flavonoids was found in the samples from Arangea and Pellaro (148.2±3.4 mg 100 mL^{-1} and 147.8±4.6 mg 100 mL^{-1}, respectively). With regard to total polyphenols significant differences in the various samples analyzed were observed, their content being between

183 180.5±2.9 mg 100 mL^{-1} and 233.4±0.5 mg 100 mL^{-1}. The Catona sample was significantly lower
184 (180.5±2.9 mg 100 mL^{-1}) compared to the other samples.

185 Table 5 shows the data collected for the flavonoid content of bergamot juice. The most abundant
186 components in bergamot juices are naringin, neoeriocitrin and neohesperedin, as reported by
187 Gionfriddo et al.;[5] Kawaii et al.; [6] Calabrò et al.;[34] Dugo et al.;[8] Gattuso et al.;[35]
188 Nogata. [9]

189 The lowest values of flavonoids identified by HPLC on the basis of retention times and
190 quantified from the peak areas compared to standards, are those collected from the areas of
191 Gallico and Palizzi.

192 Reactive oxygen species (ROS) are closely related to many pathological conditions such as
193 inflammation, tumours, cardiovascular disease, cerebral ischemia and diabetes. DPPH· and
194 ABTS· are stable free radicals, which have been widely accepted as a tool for estimating free
195 radical scavenging activities of antioxidants.[36] All samples exhibited a radical scavenging
196 activity against both radicals in a concentration-dependent manner (Table 6). The area of
197 collection influenced the DPPH radical scavenging activity with a range of IC_{50} values from
198 19.6±2.0 to 31.4±1.1 μg mL^{-1} (Melito and Palizzi samples, respectively). The sample was also
199 Melito the most effective also in the ABTS test with IC_{50} value of 17.4±1.6 μg mL^{-1} followed by
200 the Africo sample (IC_{50} value of 18.9±2.4 μg mL^{-1}). Correlation analysis revealed that the DPPH
201 assay is positively correlated with total phenol and flavonoid content. However total phenols
202 positively correlated also with ABTS data. The potential of bergamot juice to inhibit lipid
203 peroxidation was evaluated using the β-carotene/linoleic acid bleaching test, which measures the

capacity for inhibiting conjugated diene hydroperoxide formation during linoleic acid oxidation. Even in this case the Melito was sample the most active (IC_{50} values of 25.7±1.2 and 24.9±3.0 μg mL^{-1} at 30 and 60 minutes of incubation). The correlation analysis revealed that data obtained from this assay are positively correlated with vitamin C and total flavonoids, which are mainly responsible for antioxidant activity. A significant positive correlation was also observed with eriocitrin, naringin, neoeriocitrin and neohesperidin content. Previously Gardner et al.[37] reported that vitamin C was found to account for 65-100% of the antioxidant potential of *Citrus* juice.

The antioxidant potential of *C. bergamia* juice has been previously investigated by Trovato et al.[38] that found a noticeable effect on scavenging DPPH radicals with IC_{50} value of 25.01 µL. Xu et al.[39] studied the total phenolic content and antioxidant activities of fifteen *Citrus* variety juices. The total phenolic content ranged from 751.82 to 1555.49 mg $^{L-1}$ for *C. limon* and hybrid 439 (*C. reticulata* x *C. sinensis*), respectively. It was interesting that Hybrid 439 achieved the highest DPPH inhibitory activity with a percentage of inhibition of 61.62% followed by *C. sinensis* Osbeck cv Hamlin and cv Liubencheng that exhibited a DPPH· scavenging ability of 60.24 and 60.13%. Interestingly in *Citrus* investigated by Xu et al. [39] neohesperidin was found only in two varieties.

CONCLUSIONS

For all the studied characteristics there were statistically significant differences ($P < 0.05$) between juices analyzed. The purpose of this thesis was to see how the microclimate of a given

area of cultivation may influence the quality and, therefore, the chemical composition of the juice itself. Results obtained in this study are a contribution to the characterization of bergamot fruits (*Citrus bergamia* Risso) cultivated in different areas of the province of Reggio Calabria (Italy).

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340 **Table 1** Sites of collection of Citrus bergamia in province of Reggio Calabria (Italy)

Area of collection	Latitude	Longitude
Catona	38.21705	15.63691
Gallico	38.16559	15.65367
Arangea	38.10929	15.64393
Pellaro	38.02050	15.64856
Melito Porto S.	37.92072	15.78568
Palizzi	37.96705	15.98719
Africo	38.05172	16.13407

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342

343 **Table 2** Average colorimetric values of bergamot juice

344

Area of collection	**L**	**a***	**b***
Catona	1,83±0.02	0,05±0.02	0,60±0.04
Gallico	7,60±0.25	0,22±0.01	4,82±0.28
Arangea	6,69±1.35	0,10±0.11	4,47±1.46
Pellaro	4,78±0.15	0,29±0.11	2,51±0.17
Melito	1,77±0.08	0,06±0.03	0,64±0.06
Palizzi	1,95±0.11	0,21±0.07	1,09±0.11
Africo	2,21±0.03	0,21±0.06	1,32±0.06

345

346 **Table 3** Physicochemical data of bergamot juice

Area of collection	pH	Acidity g L^{-1}	RRS	Formol index	
Catona	2,84±0.00[a]	11,38±0.31[e]	8,11±0.11[d]	2,96±0.01[a]	***
Gallico	2,72±0.00[d]	12,44±0.04[d]	11,50±0.02[a]	2,53±0.02[e]	***
Arangea	2,78±0.00[c]	14,34±0.02[b]	11,61±0.48[a]	2,63±0.02[d]	***
Pellaro	2,81±0.00[b]	14,61±0.03[ab]	10,36±0.06[b]	2,75±0.0[2c]	***
Melito	2,82±0.00[ab]	14,73±0.03[a]	9,22±0.08[c]	3,63±0.01[d]	***
Palizzi	2,72±0.00[d]	13,11±0.10[c]	9,25±0.06[c]	2,91±0.02[a]	***
Africo	2,54±0.00[d]	14,83±0.06[a]	9,57±0.03[c]	2,86±0.02[b]	***

347 Values are the mean of three independent determinations ± standard deviation.

348 Means followed by different letter are significantly different (P<*0.05*).

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Table 4 Values of ascorbic acid, total flavonoid and total phenolic of Citrus Bergamia (juice)

Area of collection	Ascorbic acid mg 100mL^{-1}	Flavonoid mg 100mL^{-1}	Polyphenol mg 100mL^{-1}	
Catona	285,35±10.05^{a}	104,06±2.17^{a}	180,53±2.86^{d}	***
Gallico	89,40±2.10^{d}	51,11±2.18^{b}	209,72±1.29^{b}	***
Arangea	152,09±4.50^{c}	148,15±3.37^{a}	228,15±2.70^{a}	***
Pellaro	171,91±1.85^{b}	147,81±4.58^{c}	202,37±0.45^{c}	***
Melito	183,38±7.34^{b}	106,40±1.82^{a}	233,36±0.48^{a}	***
Palizzi	184,10±2.71^{b}	74,00±2.11^{b}	211,84±0.65^{b}	***
Africo	173,06±3.21^{b}	126,94±3.61^{c}	203,22±0.50^{c}	***

Values are the mean of three independent determinations ± standard deviation.

Means followed by different letter are significantly different (P<*0.05*).

354 **Table 5** Flavanone content (mg mL^{-1}) in bergamot juice

Area of collection	Eriocitrin mg mL^{-1}	Naringin mg mL^{-1}	Neoeriocitrin mg mL^{-1}	Neohesperedin mg mL^{-1}	
Catona	12,26±0.42^{bc}	261,81±0.88^{c}	282,47±1.70^{b}	222,24±0.85^{d}	***
Gallico	6,36±0.32^{e}	142,69±2.98^{e}	172,98±1.60^{e}	110,79±0.25^{f}	***
Arangea	14,09±0.17^{a}	281,52±2.43^{b}	296,77±1.51^{a}	230,87±0.40^{c}	***
Pellaro	14,45±0.37^{a}	295,73±1.11^{a}	279,56±1.52^{b}	244,82±0.88^{a}	***
Melito	11,76±0.25^{c}	265,18±1.33^{c}	264,63±0.89^{d}	235,66±0.81^{b}	***
Palizzi	8,07±0.22^{d}	163,18±2.80^{d}	158,59±1.05^{f}	116,63±0.39^{e}	***
Africo	12,87±0.18^{b}	286,74±0.78^{b}	272,73±1.26^{c}	116,87±0.48^{e}	***

355 Values are the mean of three independent determinations ± standard deviation.

356 Means followed by different letter are significantly different (P<*0.05*).

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358 **Table 6** Antioxidant activity of Citrus bergamia Risso

	DPPH				
IC_{50} (μg mL^{-1})	ABTS				
IC_{50} (μg mL^{-1})	β-carotene bleaching				
IC_{50} (μg mL^{-1})					
Area of collection			30 min	60 min	
Catona	20.5 ± 1.3[e]	21.8 ± 2.0[d]	32.6 ± 2.4[d]	36.7 ± 2.9[a]	***
Gallico	21.1 ± 1.0[e]	25.9 ± 1.9[c]	34.8 ± 2.7[b]	30.8 ± 2.0[e]	***
Arangea	27.3 ± 1.9[b]	32.6 ± 2.2[b]	36.8 ± 2.6[a]	35.7 ± 2.5[b]	***

Pellaro	23.9 ± 1.4[d]	25.7 ± 2.2[d]	33.5 ± 2.9[c]	34.8 ± 2.4[c]	***
Melito	19.6 ± 2.0[f]	17.4 ± 1.6[f]	25.7 ± 2.9[f]	24.9 ± 3.0[f]	***
Palizzi	31.4 ± 1.1[a]	35.6 ± 1.5[a]	33.7 ± 2.8[c]	31.8 ± 2.6[d]	***
Africo	25.7 ± 1.2[c]	18.9 ± 2.4[e]	30.0 ± 1.5[e]	35.3 ± 1.9[b]	***
Positive control					
BHT					
Ascorbic acid	5.0 ± 0.8	1.7 ± 0.3			
Propyl gallate			1.0 ± 0.04	1.0 ± 0.05	

Data are expressed as means ± S.D. (n= 3); DPPH Radical Scavenging Activity Assay; One-way ANOVA ***p <0.0001 followed by a multicomparison Dunnett's test: ***p <0.01 compared with ascorbic acid. Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$); One-way ANOVA ***p< 0.0001 followed by a multicomparison Dunnett's test: ***p< 0.01 compared with ascorbic acid. β-carotene bleaching test 30 min One-way ANOVA ***p <0.0001 followed by a multicomparison Dunnett's test: ***p< 0.01 compared with propyl gallate. β-carotene bleaching

365 test 60 min One-way ANOVA ***p <0.0001 followed by a multicomparison Dunnett's test: $^{***}p$

366 <0.01 compared with propyl gallate. Means followed by different letter are significantly different

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368 **Figure 1** Map (Calabrian, Southern Italy) showing the area where Bergamot is cultivated

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