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Investigating the in vitro hypoglycaemic and antioxidant properties of Citrus ×clementina Hort. juice

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27 **Investigating the in vitro hypoglycaemic and antioxidant properties**
28 **of *Citrus × clementina* Hort. juice**

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37

38 **Abstract**

39 *Citrus × clementina* juice obtained from fruits collected in three different areas (flood plain, hill and
40 coastal plain) was investigated for the chemical composition, radical scavenging properties (DPPH
41 and ABTS tests), and α -amylase and α -glucosidase inhibitory activity. Neohesperidin (72.96–116.50
42 mg/100 mL), hesperidin (55.24–69.52 mg/100 mL) and narirutin (7.21–12.13 mg/100 mL) are the
43 main flavonoids identified by HPLC analyses. In carbohydrate hydrolysing enzymes inhibitory
44 activity tests, samples showed higher potency against α -glucosidase. Juice from hill was the most
45 active with an IC₅₀ value of 77.79 μ g/mL. Data on the radical scavenging activity revealed the
46 following trend of potency flood plain > coastal plain > hill. These results could help farmers to select
47 fruits for different industrial purpose such as functional food and matrix to extract nutraceutical
48 products.

49

50 **Keywords:** HPLC phenolic profile · Quality parameters · Healthy properties · PCA

51

52

53 **Introduction**

54 In the last decades, there has been growing recognition of the key role of foods and beverages in
55 disease prevention and treatment. Thus, the production and consumption of functional beverage has
56 gained much importance as they provide a health benefit beyond the basic traditional nutrients [1].
57 Plants of the genus Citrus are primarily valued for their edible fruit, but they also have health
58 properties [2]. Citrus fruits are among the most important dietary sources of bioactive compounds.
59 The healthy properties of Citrus fruits have been attributed to ascorbic acid and phenolic compounds,
60 mainly to flavonoids [3]. Citrus × clementina is a hybrid between a mandarin orange and a sweet
61 orange. Clementines can be separated into 7 to 14 segments. Similar to tangerines, they tend to be
62 easy to peel. They are usually seedless when grown commercially (without cross-pollination), and
63 therefore are always known as seedless tangerines. They are typically juicy and sweet, with less acid
64 than oranges [4]. Clementines are especially appreciated for their delicious flavor, and recent years
65 have seen a great increase in the consumption of clementine juice.

66 Recently, the role of reactive oxygen species (ROS) in the pathogenesis of increasing number of
67 diseases is clarified [5]. Among them, there is diabetes mellitus as a group of metabolic diseases
68 characterized by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action,
69 or both. This condition is associated with long-term damage, dysfunction, and failure of various
70 organs, including eyes, kidneys, nerves, heart, and blood vessels. For the American Diabetes
71 Association (ADA) diabetes can be classified into the following general categories: (a) Type 1
72 diabetes or T1DM (due to β -cell destruction, usually leading to absolute insulin deficiency), (b) Type
73 2 diabetes or T2DM (due to a progressive insulin secretory defect on the background of insulin
74 resistance); (c) gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of
75 pregnancy that is not clearly overt diabetes), (d) other type. Insulin resistance is not only a key feature
76 of T2DM, but also a consequence of exposure to inflammatory cytokines such as tumour-necrosis
77 factor- (TNF-) that could activate c-Jun NH₂-terminal kinase (JNK) [6]. Moreover, ROS may
78 contribute to the long-term deterioration of insulin secretory capacity in the islet β -cell level since

79 they could affect mitochondrial ATP production that is necessary for hormone secretion.
80 Mitochondrial function also appears a critical determinant of insulin sensitivity within muscle, liver,
81 and adipose tissue. Moreover, ROS appear important in the autoimmune destruction that characterizes
82 type 1 diabetes, as well as in the pathophysiology of the long-term complications that characterize
83 both classes of diabetes [7]. Between two types of diabetes, T2DM is more prevalent than type T1DM.
84 Postprandial hyperglycaemia plays an important role in the development of T2DM so regulating
85 plasma glucose level is crucial for delaying or preventing T2DM. One of the most common
86 approaches to reduce or delay the intestinal absorption of glucose is by inhibiting carbohydrate
87 hydrolysing enzymes such as α -amylase and α -glucosidase [8]. Acarbose one of the leading inhibitors
88 of carbohydrate hydrolysing enzymes, is frequently associated with side effects such as bloating,
89 diarrhea, flatulence, cramping, and abdominal pain [9]. During the last 10 years, several works
90 investigated the potential role of phytochemicals as carbohydrate hydrolysing enzymes inhibitors. In
91 this context, we have decided to screen the phenols profile, hypoglycaemic and antioxidant potential
92 of *C. × clementina* juice collected in three different areas of growth. Therefore, the purpose of this
93 study is to (a) identify the phenols HPLC profile of juice; (b) evaluate hypoglycaemic potential and
94 the antioxidant activity of the juice (c) clarify if growth area influenced the quality parameters and
95 phytochemical content of the juice. The findings of this research study could help *C. × clementina*
96 cultivators to improve fruit quality, making it suitable for industrial products with particular reference
97 to functional juice.

98

99 **Materials and methods**

100 **Chemicals and reagents**

101 All reagents used in this study were purchased from Sigma-Aldrich S.p.a. (Milan, Italy) while solvent
102 of analytical grade was obtained from VWR International s.r.l. (Milan, Italy). Acarbose from
103 Actinoplanes sp. was obtained from Serva (Heidelberg, Germany). Vanillic acid ($\geq 99\%$ HPLC grade)
104 chlorogenic acid and gallic acid were purchased from Sigma-Aldrich Chem. Co. (Milwaukee, WI,

105 USA). Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, didymin, neohesperidin, poncirin,
106 quercetin, apigenin, sinensetin, nobiletin, and tangeritin were supplied by Extrasynthese (Genay–
107 France). Acetonitrile, formic acid and water were solvent HPLC grade, obtained from Carlo Erba
108 Reagents (Milano, Italia). Standard solutions were prepared by adding accurately weighed amount of
109 each antioxidant compound in methanol (90:10). A calibration straight for each standard was obtained
110 by analyzing the standard solution diluted at different concentrations. All solutions were filtered
111 through a 0.45 µm Millipore filter (GMF, Whatman) and injected to HPLC system for retention times
112 determination.

113

114 **Samples collection and physicochemical analysis**

115 The fruits of *C. × clementina* (Rutaceae) used in this study were collected in November 2016 in
116 Calabria (Southern Italy), Plain of Sybaris (CS) from 20 selected farms in three different areas (zone
117 A, B and C). The authentication was carried out at the Natural History Museum of Calabria and the
118 Botanic Garden, University of Calabria. In zone A, the flood plain results from the Crati and Coscile
119 rivers. The sediments, tend coarse, become finer in the less areas close to the river. The water table
120 is found below 150 cm depth. Overall, the soils in this subzone have good productive potential, while
121 demanding adequate water and nutrient management strategies. Zone B (Terrazzi antichi, Hill) is
122 placed at altitudes between 50 and 80 m above sea level. This area was characterized by evolved soils,
123 as confirmed by the red colors, with leaching and redeposition as clay pedogenetic process. The clay
124 increase along the profile has particularly affected the hydrological and chemical properties. Zone C
125 (Coastal plain) comprises the central part of the Plain of Sybaris, near the mouth of the river Crati,
126 characterized by elevations between 0 and 10 m above sea level, with depressed areas behind the
127 dunes. Reclaimed in the first half of the last century, the area currently has the water table in balance
128 with the artificial drainage system. The substrate consists of coarse sediments with alkaline reaction
129 soils, Franco-sandy becoming mostly sandy at about 110 cm deep. The low water retention capacity
130 and low cation exchange capacity make it advisable to adopt adequate water management strategies

131 and nutrient. *C. × clementina* requires a mild climate, as constant as possible during the growing
132 season, and is particularly sensitive to temperature changes, especially those caused by cold winds
133 that dry the twigs. In the plain, the summer temperatures reach a maximum value of 40 °C, while
134 winter temperatures are around 4 °C with differences of 2–4 °C above the hill. To create appropriate
135 temperature conditions during the winter period, plants are covered with dark green plastic nets.
136 Fruits were randomly harvested from 30 healthy homogeneous trees per grove. Twenty-five fruits for
137 each area of growth (A, B, and C) were collected and examined for integrity and absence of insect
138 and dust contamination. Physical characteristics of the fruits such as fruit weight (g), equatorial
139 diameter (cm), longitudinal diameter (cm), fruit firmness (g/0.5 cm²), peel thickness (mm), total
140 seeds per fruit and amount of extracted juice (%) were determined. Samples were freeze-dried and
141 stored at –20 °C until analysis. The total nitrogen content, moisture content, ash content, fat content,
142 crude fiber content, total carbohydrates, minerals, and energy values were evaluated [10].
143 *C. × clementina* fruits were squeezed and the juice was centrifuged and filtered to determine the
144 following analyses: color of fresh juice was measured at 25 °C using a Konica Minolta CM-700/600d
145 spectrophotometer (Konica Minolta Sensing, Japan). Data were expressed as higher saturation of
146 color or chroma (C*). Total soluble solids (TSS) were determined using a digital refractometer PR-
147 201α (Atago, Tokyo, Japan), previously calibrated at 20 °C and the results are expressed as degrees
148 Brix; The pH was measured at ambient temperature with a pH meter (Model Basic 20, Crison)
149 previously calibrated with standard solutions pH 4 and pH 7; Total acidity (TA) was determined using
150 the International Federation of Fruit Juice producers test (IFU): a potentiometric titration of the
151 acidity of the juice, with a solution of 0.25 N NaOH up to pH 8.1. The results were expressed as g/L
152 of anhydrous and hydrate citric acid. Ascorbic acid was determined using the International Federation
153 of Fruit Juice producers test (IFU): a potentiometric titration of the acidity of the juice, with a solution
154 of 2,6-dichloroindophenol [11]. All determinations above described were made in triplicate.

155

156 **HPLC analysis of juice**

157 C. × clementina juice was pre-treated with centrifuge (4000 rpm for 20 min), then filtered by using
158 membrane filter (0.45 µm). Separation of phytochemicals was made by using a HPLC apparatus
159 equipped with Phenomenex C18 column (150 mm × 3 mm) according to the official methodologies
160 of the International Federation of Fruit Juice producers [15]. Two buffers were used as solvent system
161 (A: Acetonitrile/Water/phosphoric acid (70:26:4) and B: Potassium Dihydrogen Phosphate at pH
162 3.5). The gradient program was as follows: starting condition, 85% A, 15% B; 5 min, 70% A, 30%
163 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.
164 The column was operated at 25 °C and flow rate was 1 mL/min. The chromatogram was monitored
165 at $\lambda = 287$ nm. Identification of compounds was performed by comparing their retention time with
166 those of standards, and confirmed with characteristic spectra using the photodiode array detector.

167

168 **Carbohydrate hydrolysing enzymes inhibition study**

169 Modulation of hyperglycaemia is an important tool in the management of the diabetic patient. α -
170 Amylase is an endoglucanase which hydrolyse the internal α -1,4 glucosidic linkages in starch while
171 α -glucosidase is one of the glucosidases located in the brush border surface membrane of intestinal
172 cells. Both enzymes are involved in carbohydrates digestion and absorption. For the above reason,
173 both enzymes have been recognized as therapeutic targets for modulation of postprandial
174 hyperglycaemia in T2DM [8]. A starch solution, α -amylase (EC 3.2.1.1) solution, and colorimetric
175 reagent were prepared. Both control and juice were added to starch solution and left to react with
176 enzyme at room temperature for 5 min [12]. The absorbance was read at 540 nm. The enzyme
177 inhibition (%) was obtained by the following equation:

$$178 \text{ \%Inhibition} = 100 - \left(\frac{[\text{Maltose}]_{\text{test}}}{[\text{Maltose}]_{\text{control}}} \times 100 \right) \pm \text{S.D.}$$

179 In the α -glucosidase inhibition test, a maltose solution, α -glucosidase solution (EC 3.2.1.20) and o-
180 dianisidine (DIAN) solution were prepared [12]. A mixture of juice maltose solution and enzyme
181 were left to incubate at 37 °C for 30 min. Then, perchloric acid was added and mixture was
182 centrifuged. The supernatant was collected and mixed with DIAN and PGO and left to incubate at 37

183 °C for 30 min. The absorbance was read at 500 nm. The α -glucosidase inhibition (%) was calculated
184 by the following equation:

$$185 \quad \% \text{Inhibition} = 100 - \left(\frac{[\text{Glucose}]_{\text{test}}}{[\text{Glucose}]_{\text{control}}} \times 100 \right) \pm \text{S.D.}$$

186

187 **Radical scavenging activity**

188 Oxidative stress is a normal phenomenon in the body. Under normal conditions, the physiologically
189 important intracellular levels of reactive oxygen species (ROS) are maintained at low levels by
190 various enzyme systems participating in the in vivo redox homeostasis [13]. The evaluation of
191 antioxidant activity is context-dependent. In recent years, many different methods have been
192 proposed for the evaluation of antioxidant activity. Most of them are based on the measurement of
193 the relative abilities of antioxidants to scavenge radicals in comparison with the antioxidant potency
194 of a standard antioxidant compound.

195 The antioxidant activities of *C. × clementina* juice samples were assessed by using in vitro assays
196 namely ABTS and DPPH.

197 ABTS assay was applied by using the previously published methodology with slight modifications
198 [14]. ABTS radical cation (ABTS⁺) was mixed with potassium persulphate and left in the dark for
199 12 h before use. The ABTS⁺ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at λ
200 = 734 nm by using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. A mixture of juice and
201 diluted ABTS⁺ solution was prepared and after 6 min the absorbance was measured at 734 nm. The
202 scavenging ability of the juice was calculated according to the following equation: ABTS scavenging
203 activity (%) = $\left[\frac{A_0 - A}{A_0} \right] \times 100$ where A_0 is the absorbance of the control reaction and A is the
204 absorbance in the presence of samples.

205 DPPH radical scavenging assay was previously described by Loizzo et al. [14]. An ethanol solution
206 of DPPH radical (DPPH[·]) at concentration of 1.0×10^{-4} M was mixed with juice. The reaction
207 mixtures left in the dark for 30 min. The absorbance was measured at $\lambda = 517$ nm against blank

208 without DPPH. The DPPH radical scavenging activity (%) was calculated according the following
209 equation:

210 % DPPH radical-scavenging= $[1 - (\text{sample absorbance with DPPH} - \text{sample absorbance without}$
211 $\text{DPPH/control absorbance})] \times 100$

212 Relative antioxidant capacity index (RACI) calculation

213 Relative antioxidant capacity index (RACI) is a statistical application to integrate the antioxidant
214 capacity values generated from different in vitro methods [15].

215 The standard scores were derived from data from different chemical methods with no unit limitation
216 and no variance among methods. Therefore, it can be used as an integrated approach to evaluate and
217 compare the antioxidant capacity of different samples. Thus, data obtained from TPC, TCC, ABTS
218 and DPPH tests were used to calculate a RACI value for juice.

219

220 **Soil and leaf analysis**

221 Soils were sampled in March, before the annual application of fertilizers, using a manual drill. In each
222 grove, four soil specimens were collected at depths of 0–30 cm and 30–60 cm and mixed to form a
223 single sample. Total CaCO₃ (%) was determined using a calcimeter [16]. Organic matter (%) was
224 assessed using the Walkley–Black method, available P (µg/g) was determined by the Olsen method
225 while Kjeldahl method was used for total N (%) [16, 17]. Atomic absorption spectrophotometry was
226 applied to determine exchangeable Ca, K, Na and Mg cations (µg/g) [16]. The pH was also measured
227 (PH211 pH meter, HANNA Instruments). The leaves were picked in October when the level of
228 elements was stable and before fertilizers were applied. The leaves collected for analysis were 5–7
229 months old. Foliar analysis was performed on 30 leaves from the index trees picked from non-fruit
230 bearing terminal shoots of the year's spring flush [18]. Determination of total nitrogen was made as
231 previously reported while inductively spectrometer plasma (ICP) technique was used for macro and
232 micro-elements [19].

233

234 **Statistical analysis**

235 All experiments were carried out in triplicate. Data are expressed as mean \pm standard deviation (S.D.).
236 The concentration giving 50% inhibition (IC₅₀) was calculated by nonlinear regression with the use
237 of Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The
238 dose–response curve was obtained by plotting the percentage inhibition versus concentration.
239 Differences within and between groups were evaluated by one-way analysis of variance test
240 (ANOVA) followed by a multicomparison Dunnett’s test compared with the positive control. To
241 achieve the objectives set, the data obtained were processed using statistical procedures that tended
242 to highlight any significant relationship of nutrients in the leaves and in the physico-chemical
243 parameters of soil with the phenolics identified in C. \times clementina juice. Studies of the Pearson’s
244 correlation coefficient (r) and linear regression, assessment of repeatability, calculation of average
245 and relative standard deviation was performed using Microsoft Excel 2010 software. Moreover,
246 differences between zone A, B and C were underlined. Similarities between clementine juices and
247 differences between three geographical areas (A, B, C) were studied. One-way ANOVA (Tukey’s
248 test) and Principal Component Analysis (PCA) were applied by SPSS software for Windows, version
249 15.0 (Chicago, IL, USA).

250

251 **Results and discussion**

252 *Fruits quality parameters*

253 Fruit carpometric parameters displayed some statistically significant differences (Table 1). In
254 particular, fruits collected in the flood plain were characterized by a lower weight (87.19 g) and lower
255 fruit firmness (determined on a portion of peel and ‘albedo’). Several differences were evidenced also
256 in equatorial and longitudinal diameter. Fruits collected in coastal plain are characterized by the
257 highest number of seed (6.31 seeds per fruits). Despite the weight differences among the fruits, the
258 difference in percentage yield of juice is minimal 47.32 vs 49.60% for zone A and C, respectively.

259

260 *C. × clementina* juice analysis

261 Each investigated sample give a percentage of juice ranging from 45.61 to 49.60% for zone B and C,
262 respectively, (Table 2). The *C. × clementina* juice quality parameters, including pH, total soluble
263 solids, total acidity, ascorbic acid content, and color were investigated. The values of total acidity
264 ranged from 0.35 to 0.88 g citric acid/100 mL for juice obtained from fruits collected in zone B and
265 C, respectively. The highest total soluble solids (11.63 °Brix) were detected with juice obtained from
266 fruits collected in coastal plain. A pH ranging from 3.50 to 3.76 was measured. Moreover, juice from
267 fruits collected in coastal plain is rich in ascorbic acid (66.25 mg/L). No significant differences were
268 evidenced in chroma value (C*). Samples are rich also in phenols and carotenoids with a total phenol
269 content ranging from 29.74 to 44.20 mg GAE/100 mL for zone A and C, respectively, and from 42.89
270 to 75.45 mg β-carotene/100 mL for A and B, respectively. Lower values of vitamin C were detected
271 by Bermejo et al. [20] that screened 15 mandarin cultivars. Among them, Arrufatina, Loretina and
272 Fina displayed the highest values of total vitamin C, followed by the hybrid Ellendale.
273 Previously, Al-Mouei and Choumane [21] determined the quality parameters of twelve Citrus
274 varieties namely common mandarin, mandalina, clementine, Nova, Carvalhal, Dancy, Klimntard,
275 Fortune, Ortanique, Minneola, Ponkan and Satsuma growing in Syria. Among all investigated
276 varieties group, Ortanique had the highest juice content (56.1%) while common mandarin had the
277 lowest content (37%). The TSS in mandarin group ranging from 9.5 to 13.9 °Brix. Our results on pH
278 measurement are in line with those reported by the authors that found a pH value of 3.5 for Clementine
279 while Fortune variety showed the lowest value 2.67. Great variability on total acidity was found also
280 for mandarin group with Minneola that contained high TA value (1.62 g citric acid/100 mL) while
281 Nova has lowest acidity value (0.49 g citric acid/100 mL).

282

283 Juices from Cadoux, Monreal, St. Martin, Merme, Cheylard, and Rocamora clementine cultivar and
284 one mandarin fruit cultivar were investigated for quality parameters and antioxidant potential [22].
285 The TA of investigated juices ranged from 4.61 to 7.47 g of citric acid/L for clementine cv. Merme

286 and mandarin, respectively, with a pH varied between 3.91 and 3.68 for the same cultivars,
287 respectively. Ascorbic acid content was lowest in clementine St Martin cv. (35.98 mg/100 mL).
288 Mineral analysis showed that all investigated cultivars are rich in potassium.

289

290 Fruit juices of Safor [(*C. clementina* × *C. tangerina*) × (*C. unshiu* × *C. nobilis*)], Garbí [(*C. clementina*
291 × *C. tangerina*) × (*C. reticulata* × *C. sinensis*)], Fortune (*C. clementina* × *C. tangerina*), Kara (*C.*
292 *unshiu* × *C. nobilis*) and Murcott (*C. reticulata* × *C. sinensis*) were investigated for juice quality
293 parameters including juice yield, TA and TSS [23]. A percentage of juice ranging from 43.3 to 59.9%
294 was found for Murcott and Fortune, respectively. Garbí juice displayed the greatest total soluble
295 solids values whereas Murcott presented the lowest values. With respect to the acidity, all values are
296 higher than those found for Calabria clementine juice with similar total acidity values for Garbí,
297 Fortune and Kara juice. Similar values of juice yield, TSS and TA were evidenced also in Wase-
298 Satsuma, Satsuma, Ponkan, Bendizao, Manju, new variety Hybrid 439 and Zhuhong mandarins from
299 China [24]. Manju variety achieved the highest yield (60.74%) while Hybrid 439 had the highest TSS
300 value (14.92%). Ascorbic acid content of Hybrid 439 agrees with those reported for our samples.

301

302 The juice of thirteen cultivars of *C. clementina* was investigated for its quality parameters and radical
303 scavenging potential [25]. A pH values ranging from 2.60 to 3.58 Mandalate and Spinoso cultivars,
304 respectivel, were found. In line with our results, samples showed a total acidity ranging from 9.25 to
305 12.12 °Brix for Rubino and RA92 cultivars, respectively. A great variability was found in ascorbic
306 acid content with values from 205.85 to 643.73 mg/L for Mandalate and Fedele cultivars,
307 respectively. The high content of healthy compounds was confirmed also in this study since authors
308 reported a mean content of β -carotene of 13.58 mg/L. Similar results were also obtained by RA 85
309 and in RA 133 cultivars by Dhuique-Mayer et al. [26] and in two new mandarin-like hybrids (*C.*
310 *clementina* × *C. sinensis*) by Rapisarda et al. [27].

311

312 Evolution of juice yield, TSS, TA, and vitamin C content of new pigmented Citrus hybrid namely
313 Omo-31 and those of its parent clementine cv. Oroval (*C. clementina*) and Moro orange (*C. sinensis*)
314 were investigated during fruit maturation [28]. Results clearly evidenced that juice yield, TSS and
315 TA values of new pigmented Citrus hybrid were similar to those of the Moro orange. No differences
316 were observed among the three genotypes on vitamin C content at maturity stage (~47–48 mg/100
317 mL of juice). HPLC–DAD phenols profile evidenced the presence of flavonoids. These compounds
318 are known as healthy compounds [29]. Thirteen flavonoids namely eriocitrin, neoeriocitrin, narirutin,
319 naringin, hesperidin, didymin, neohesperidin, poncirin, quercetin, apigenin, sinensetin, nobiletin, and
320 tangeritin were selected as markers and quantified in *C. × clementina* juice. Data are reported in Table
321 5. Among identified constituents, the flavanone glycoside neohesperidin (116.50–72.96 mg/100 mL
322 for zone A and C, respectively) was the main abundant compound followed by the flavanone
323 aglycones hesperidin (69.52–55.24 mg/100 mL for zone C and A, respectively). However, several
324 differences were displayed in fact neohesperidin was 1.6 times higher in sample from flood plain in
325 comparison to hill. Significant amounts of narirutin (7.21–12.13 mg/100 mL) were also detected. The
326 flavanone-O-glycosides didymin and eriocitrin are mainly contained in juice from fruits collected in
327 the flood plain area. Interestingly, naringin was not detected in sample from coastal plain area. The
328 trend hesperidin > narirutin > didymin was confirmed also by Bermejo et al. [20]. for all investigated
329 samples except Murcott and Murta cultivars. Chlorogenic acid, vanillic acid and gallic acid were also
330 quantified. Chlorogenic acid was the main abundant compound with particular reference to juice
331 obtained from fruits from flood plain area (3.59 µg/mL). Gattuso et al. [30] reported the chemical
332 composition of *C. clementina* juice composition in which high content hesperidin (9.9 mg/100 mL),
333 followed by narirutin (4.64 mg/100 mL) were found. A similar trend was observed also by Rapisarda
334 et al. [28] for clementine collected in Acireale, Sicily. In the same study, the juice of hybrid Omo-
335 narirutin was not the second abundant flavanone glycoside after hesperidin. Higher values were
336 reported by Milella et al. [26] who found a hesperidin content from 63.98 to 165.88 mg/L for Etna
337 hybrid and Rubino cultivar, respectively, in agreement with those reported by Kanaze et al. [31]

338 Previously Xu et al. [24] quantified narirutin, hesperidin, naringin, and neohesperidin in different
339 Citrus varieties (Wase-Satsuma, Satsuma, Ponkan, Bendizao, Manju, new variety Hybrid 439 and
340 Zhuhong). Interestingly, in spite of our data in which the flavanone glycoside neohesperidin
341 represents the main abundant compound in Citrus juice from China is not detected together with
342 naringin. On the contrary, Nogata et al. [32] reported the presence of rutin in significant amount in
343 *C. clementina* juice.

344

345 **Carbohydrate hydrolysing enzyme activities**

346 The inhibition of carbohydrate hydrolysing enzymes α -amylase and α -glucosidase was investigated
347 and results are reported in assays as reported in Table 3. All investigated samples could inhibit both
348 enzymes in a concentration-dependent manner, the most promising activity was found against α -
349 glucosidase enzyme (Fig. 1). In particular, juice from hill exhibited the highest inhibitory activity
350 with IC₅₀ value of 77.79 μ g/mL, followed by coastal plain juice (IC₅₀ value of 93.31 μ g/mL) ($p <$
351 0.0001 , $\alpha = 0.05$). No significant differences were evidenced against α -amylase with IC₅₀ values
352 ranging from 226.69 to 243.24 μ g/mL for hill and coastal plain juice, respectively ($p < 0.0001$, $\alpha =$
353 0.05). Pearson's correlation coefficient was found positive for neohesperidin and α -glucosidase ($r =$
354 0.57), and for hesperidin and α -amylase ($r = 0.40$). The efficacy of several Citrus fruit extracts/juice
355 in the management of diabetes is supported by conclusive evidence from in vitro and in vivo models
356 [33, 34, 35, 36, 37].

357 Fresh juice from fruits *C. hystrix* and *C. maxima* showed in vitro hypoglycaemic effect with inhibition
358 of 75.55–79.75% of α -amylase and 70.68–72.83% of α -glucosidase enzyme [38]. Moreover, *C.*
359 *paradisi* juice significantly reduced rapid blood glucose levels without any effect on 1.5-h plasma
360 insulin levels [39]. More recently, Mollace et al. [40] demonstrated that bergamot juice extract
361 administered for 30 days in Wistar rats and in 237 patients both characterized by hyperlipaemia
362 associated or not with hyperglycaemia, is able to induce a significant decrease in blood glucose level
363 in both rats and patients.

364 Among Citrus phytochemicals, flavonoids are mainly involved in the management of T2DM. They
365 are able to (a) inhibit carbohydrate hydrolysing enzymes [9, 35]; (b) inhibit sodium-dependent
366 glucose transporter 1 (SGLT1); (c) stimulate insulin secretion; (d) reduce hepatic glucose output; and
367 (e) enhance insulin-dependent glucose uptake [41, 42]. In particular, the main abundant flavonoids
368 of *C. × clementina* juice inhibited both α -amylase and α -glucosidase in a concentration-dependent
369 manner, and were more active than the prescribed drug acarbose. The most active was didymin that
370 showed an IC₅₀ value of 4.20 μ M against α -glucosidase, followed by naringin (IC₅₀ value of 10.33
371 μ M), narirutin (IC₅₀ value of 14.30 μ M), and hesperidin (IC₅₀ value of 15.89 μ M). This last
372 flavanone glycoside was able to inhibit α -amylase with an interesting IC₅₀ value of 26.04 μ M.
373 Among flavonoids identified in clementine juice the most active against α -amylase was neoeriocitrin
374 with an IC₅₀ value of 4.69 μ M [35]. Previously, Shen et al. [43] studied the effect of hesperidin,
375 naringin, neohesperidin, and nobiletin on amylase-catalyzed starch digestion, pancreatic α -amylase
376 and α -glucosidase, and glucose utilization. All investigated flavonoids are able to inhibit amylase-
377 catalyzed starch digestion. Neohesperidin and naringin principally inhibited amylose digestion,
378 whereas hesperidin, inhibited both amylose and amylopectin digestion.

379 More recently, Jia et al. [44] have demonstrated that neohesperidin, the main abundant compound in
380 clementine juice significantly reduced serum glucose and glycosylated serum protein in vivo. All
381 these evidences demonstrated that this flavonoid could prevent the progression of hyperglycaemia in
382 T2DM patients by a complex mechanism that involves the binding of starch, an increase of glycolysis
383 and glycogen concentration, the lower level of gluconeogenesis, an elevating oral glucose tolerance
384 and insulin sensitivity, and decreasing insulin resistance. Moreover, the hydrolysis of starch by
385 amylase is inhibited by vitamin C alone and vitamin C–Cu complex, the latter exerting greater
386 inhibition [45]. In this study, a positive correlation was found between vitamin C and both α -amylase
387 and α -glucosidase with *r* values of 0.98 and 0.55, respectively. A positive Pearson's correlation
388 coefficient was found also between total phenols content and α -amylase (*r* = 0.58).

389

390 **Radical scavenging activity**

391 Currently, several research studies supported the role of oxidative stress in the pathogenesis of
392 diabetes. Free radical formation in diabetic patients by non-enzymatic glycation of proteins, glucose
393 oxidation and increased lipid peroxidation leads to damage of enzymes, cellular machinery and
394 increased insulin resistance due to oxidative stress. In particular, studies support the role of
395 hyperglycaemia in the generation of oxidative stress leading to endothelial dysfunction in blood
396 vessels of diabetic patients [46].

397 Herein we report the radical scavenging activity evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH)
398 radical and the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. In this
399 study, the analysis between phenolic compounds and antioxidant activity revealed a positive
400 correlation between both total phenols and carotenoids content, DPPH, and ABTS tests with r values
401 of 0.99 and 0.96, respectively. Samples showed radical scavenging activity in a concentration-
402 dependent manner in both assays. Juice obtained from fruits collected in flood plain exhibited the
403 highest radical potential with IC₅₀ values of 26.86 and 47.92 µg/mL for DPPH and ABTS test,
404 respectively (Table 4) ($p < 0.0001$, $\alpha = 0.05$). The RACI of each juice sample was calculated as the
405 mean of standard scores transformed from the raw data generated with different antioxidant methods.
406 The difference in units and variances in the raw data had no influence on the RACI. Stepwise
407 regression between RACI and different chemical methods revealed that (a) each of the assays was
408 selected as a significant variable with no single applied method being removed, (b) each method
409 contributed the same weight in building RACI, and (c) the regression was highly significant ($r = 1$, p
410 < 0.001). Therefore, RACI of each juice is a scientific combination of data from different antioxidant
411 methods with no unit limitation and no variance among methods, and makes comparison of matrix
412 antioxidant capacity probable and possibly more accurate. Based on RACI, the following antioxidant
413 rank of order has been found: zone B > zone C > zone A (Fig. 2). This trend clearly evidenced that
414 juice from zone B had the highest antioxidant potential.

415 Previously, Boudries et al. [22] reported the strong radical scavenging potential of clementine with
416 IC₅₀ values from 1.14 to 1.91 mg/mL for Merme and St Martin cultivars, respectively. No
417 significant differences in DPPH· radical scavenging ability were found in Safor, Fortune, Kara,
418 Murcott juice with the only exception of Garbí juice. This evidence is probably due to the lower level
419 of ascorbic acid in Garbí mandarin (21.19 mg/100 mL) [23]. A great variability in radical scavenging
420 potential was found by Xu et al. [24] that showed a percentage of inhibition of DPPH· radical from
421 23.69 to 61.62% for Manju and hybrid 439, respectively, at maximum concentration tested. The
422 radical scavenging potential of clementine mandarins was confirmed also by Russo et al. [34] that
423 found as Caffin, Fedele, Ragheb and RA89 juice methanol extracts from fruits collected in Metaponto
424 (Basilicata region, Italy) showed the highest ABTS radical scavenging activity with values from 23.77
425 to 25.52 mg Trolox equivalent/100 mL of juice, respectively.

426 Several identified compounds can scavenge DPPH radical. In particular, the main abundant
427 constituent neohesperidin showed in DPPH test an IC₅₀ value of 13.40 mM.

428 Values of 16.54, 36.16 and 45.30 mM were recently reported for hesperidin, didymin and narirutin
429 by Tundis et al. [35]. The antioxidant effect of Citrus mandarin varieties was confirmed also through
430 in vivo study by Codoñer-Franch et al. [36]. Diet supplementation of hypercholesterolemic children
431 with 500 mL/day of pure (100%) mandarin juice (*C. clementina*) for 28 days results in a strong
432 reduction of plasma biomarkers levels of oxidative stress, whereas the plasma antioxidants vitamin E
433 and C and intra-erythrocyte glutathione level were significantly increased.

434

435 **Soil and leaves parameters**

436 Analysis of soil in the different areas of collections evidenced that almost all physical and chemical
437 parameters were significantly different (Table 5). The soil in the plain at sea level was characterized
438 by a high sand content (64.22%), and by lower P₂O₅ and K₂O. In contrast, the soil in the area in the
439 flood plain presented a lower content of total lime (6.89%) in comparison with the plain at sea level
440 (8.96%). Leaf analysis was used to determine the nutrient status of the tree and to understand the

441 nutritional requirements of *C. × clementina* tree. As evidenced in Table 6 all investigated leaf shows
442 an optimal content micro and macro-nutrients. The nitrogen content was particularly high in zone B
443 (3.39%), probably caused by the higher intake of nitrogen fertilizer. Significant differences were
444 noted for Ca with higher levels in the flood plain area. Significantly different was also data regarding
445 P since it is three times higher in hill (zone B) respect zone A and C.

446

447 **Principal component analysis (PCA)**

448 Results of chemical composition and functional properties in relation to nutritional status,
449 environmental and soil parameters, were analyzed by multivariate principal component analyses
450 (PCA) method (a data matrix was created using the geographical areas as column and the chemical
451 parameters as lines).

452 PCA results revealed that the first two principal components explained total variance completely
453 100%. PC 1 was responsible for 53.19% of the data variability. The second principal component
454 described 46.81% of the total variance. Figure 3 shows PC1 and PC2 score plot, with points
455 representing different geographical areas. Juice extracted from fruits harvested in zone A was situated
456 in the lower left quadrant, showing negative correlation with PC1. The area B juice (lower right
457 quadrant) showed a positive correlation with PC1, while the area C juice (upper left quadrant) position
458 suggested a positive correlation with PC2. From the analysis of variable loadings, it has been observed
459 that most variables were located at the upper left and right quadrants of PCA plot (Fig. 3), indicating
460 positive correlation with PC1 and PC2. Figure 4 shows also the correlation with the chemical
461 variables. We can observe from the graph that the antioxidant activity values obtained with both the
462 method of DPPH that with the ABTS, show a positive correlation with the content of β -carotene,
463 tangeritin, naringin, neoeriocitrin, narirutin. They are negatively correlated with poncirin, eriocitrin,
464 quercetin, didymin, ascorbic acid. α -Glucosidase show a positive correlation with quercetin,
465 eriocitrin, apigenin, poncirin, while α -amylase show a strongly positive correlation with ascorbic acid,

466 didymin, and poncirin. Soil formed by active lime shows a positive correlation with the content of
467 gallic acid, vanillic acid, chlorogenic acid, naringin and tangeritin.

468

469 **Conclusion**

470 Herein, we report the investigation of chemical profile, carbohydrate hydrolysing enzymes inhibitory
471 activity and antioxidant properties of *C. × clementina* juice. The influence of area of collection,
472 environmental parameters, physico-chemical parameters of the soil and nutrients in the leaves on
473 juice chemical composition was also analyzed. Place of fruits collection positively influenced the
474 quality and bioactivity of the juice. In particular, juice obtained from fruits collected in the hill was
475 characterized by a higher content of bioactive compounds and α -glucosidase inhibitory property.
476 Collectively, these findings could help farmers to improve fruit quality, making it suitable for
477 different industrial purposes such as functional foods or nutraceuticals.

478

479 **Abbreviations**

480 **ABTS:** 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

481 **ADA:** American Diabetes Association

482 **DPPH:** 2,2-diphenyl-1-picrylhydrazyl

483 **JNK:** C-Jun NH₂-terminal kinase

484 **PCA:** Principal component analysis

485 **ROS:** Reactive oxygen species

486 **T1DM:** Type 1 diabetes

487 **T2DM:** Type 2 diabetes

488 **TNF- α :** Tumour-necrosis factor- α

489

490

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599

600

601 **Table 1** Nutritional analysis and minerals in *C. × clementina* pulp

Nutritional constituents	Zone A (g/100 g)	Zone B (g/100 g)	Zone C (g/100 g)
Ash	0.43	0.44	0.47
Fat	0.15	0.15	0.16
Protein	0.85	0.88	0.89
Fiber	1.78	1.76	1.86
Carbohydrates	12.14	12.02	12.35
Energy	53 kcal/100 g	53 kcal/100 g	54 kcal/100 g
Minerals	Content (mg/100 g)	Content (mg/100 g)	Content (mg/100 g)
Phosphorus	19	21	22
Potassium	177	179	185
Calcium	30	32	32
Magnesium	14	10	14

Zone A: Flood plain; Zone B: Hill; Zone C: Coastal plain

602

Nutritional constituents	Zone A (g/100 g)	Zone B (g/100 g)	Zone C (g/100 g)
Ash	0.43	0.44	0.47
Fat	0.15	0.15	0.16
Protein	0.85	0.88	0.89
Fiber	1.78	1.76	1.86
Carbohydrates	12.14	12.02	12.35
Energy	53 kcal/100 g	53 kcal/100 g	54 kcal/100 g
Minerals	Content (mg/100 g)	Content (mg/100 g)	Content (mg/100 g)
Phosphorus	19	21	22
Potassium	177	179	185
Calcium	30	32	32
Magnesium	14	10	14

604

605 Table 2. Fruit quality characteristics

Parameters	Zone A	Zone B	Zone C
Fruit weigh (g)	87.19 ± 3.21	109.67 ± 5.9	146.61 ± 9.1
Equatorial diameter (cm)	4.71 ± 0.72	4.42 ± 0.80	4.93 ± 0.79
Longitudinal diameter (cm)	4.54 ± 0.81	6.23 ± 0.85	6.72 ± 0.98
Fruit firmness (g/0.5 cm ²)	300.56 ± 13.98	402.12 ± 12.56	423.92 ± 14.31
Peel thickness (mm)	11.23 ± 0.51	11.25 ± 0.27	12.29 ± 0.40
Total seeds per fruit	4.21 ± 0.36	5.54 ± 0.22	6.31 ± 0.38
Juice (%)	47.32 ± 5.07	45.61 ± 6.40	49.60 ± 8.42
Juice (pH)	3.67 ± 0.07	3.50 ± 0.05	3.76 ± 0.08
Acidity (g/100 mL)	0.53 ± 0.03	0.35 ± 0.05	0.88 ± 0.02
°Brix	10.90 ± 0.02	9.30 ± 0.01	11.63 ± 0.02
Ascorbic acid (mg/100 mL)	65.92 ± 3.46	60.40 ± 2.26	66.25 ± 3.88
TPC (mg GAE/100 mL)	29.74 ± 0.12	32.16 ± 0.18	44.20 ± 1.28
TCC (mg β-carotene/100 mL)	42.89 ± 1.83	75.45 ± 0.79	49.69 ± 1.56
C* peel	51.78 ± 1.80	46.89 ± 1.45	49.86 ± 1.80
C* pulp	20.94 ± 1.23	20.62 ± 1.16	20.86 ± 1.25

607

608

609

610 **Table 3.** HPLC analysis of selected markers of *C. × clementina* juice. Results are expressed as mg/100 mL

Selected markers	Zone A	Zone B	Zone C 611
Apigenin	0.095 ± 0.01	0.055 ± 0.07	0.078 ± 0.01
Chlorogenic acid	3.59 ± 0.45	2.88 ± 0.36	2.07 ± 0.27
Didymin	5.65 ± 0.84	3.65 ± 0.44	5.36 ± 0.72
Eriocitrin	1.27 ± 0.02	1.14 ± 0.11	1.19 ± 0.04
Gallic acid	1.25 ± 0.03	1.77 ± 0.05	0.85 ± 0.06
Hesperidin	55.24 ± 2.53	60.39 ± 4.21	69.52 ± 3.88
Naringin	1.97 ± 0.01	n.d.	1.14 ± 0.08
Narirutin	9.91 ± 0.14	12.13 ± 0.92	7.21 ± 0.90
Neohesperidin	2.25 ± 0.14	3.5 ± 0.08	3.2 ± 0.07
Neohesperidin	116.50 ± 5.63	107.47 ± 7.29	72.96 ± 6.49
Nobiletin	0.10 ± 0.01	0.09 ± 0.01	0.15 ± 0.01
Poncirin	2.15 ± 0.01	1.28 ± 0.02	1.88 ± 0.03
Quercetin	0.60 ± 0.02	0.26 ± 0.03	0.25 ± 0.02
Sinensetin	0.006 ± 0.01	0.008 ± 0.01	0.008 ± 0.05
Tangeritin	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01
Vanillic acid	1.87 ± 0.02	1.91 ± 0.02	0.44 ± 0.01

612

613 **Table 4 Hypoglycaemic activity and radical scavenging activity of *C. × clementina* juice (IC₅₀ µg/mL)**

Assay	Zone A	Zone B	Zone C
Hypoglycaemic			
α-Amylase	238.86 ± 0.8***	226.60 ± 1.7***	243.24 ± 2.3***
α-Glucosidase	200.92 ± 5.5***	77.79 ± 3.7***	93.31 ± 3.2***
Radical scavenging			
DPPH	26.86 ± 0.8***	47.98 ± 1.2***	32.35 ± 0.9***
ABTS	47.92 ± 2.5***	65.21 ± 3.9***	56.11 ± 3.9***

614

615 **Table 5 Physical and chemical parameters of soils in the zone A, B and C**

Parameters	Zone A	Zone B	Zone C
Clay (%)	18.29 ± 2.10	20.45 ± 2.37	21.31 ± 2.87
Silt (%)	19.46 ± 3.12	21.38 ± 1.91	26.72 ± 2.96
Sand (%)	57.66 ± 4.15	57.31 ± 3.88	64.22 ± 3.45
Total N (%)	1.20 ± 0.23	1.05 ± 0.38	1.07 ± 0.21
P ₂ O ₅ (mg kg ⁻¹)	128.07 ± 9.89	188.07 ± 11.64	145.09 ± 10.24
K ₂ O (mg kg ⁻¹)	43.38 ± 10.59	56.38 ± 9.15	36.28 ± 9.32
Na (mg kg ⁻¹)	29.27 ± 14.94	24.68 ± 10.86	30.18 ± 11.97
MgO (mg kg ⁻¹)	183.38 ± 18.93	213.13 ± 17.28	215.10 ± 15.29
TOC (%)	0.98 ± 0.20	1.05 ± 0.31	1.03 ± 0.20
S.O. (%)	1.69 ± 0.49	1.99 ± 0.56	1.99 ± 0.32
Total lime (CaCO ₃ %)	6.89 ± 1.08	7.96 ± 0.28	8.96 ± 0.78
Active lime (%)	3.35 ± 1.26	3.54 ± 0.67	3.00 ± 0.44
pH	9.31 ± 0.24	8.34 ± 0.29	6.25 ± 0.20

Electrical conductivity (mS/cm 25 °C)	1.05 ± 0.30	1.03 ± 0.22	1.07 ± 0.21
C.S.C. (meq/100 g soil)	10.86 ± 1.38	13.18 ± 0.61	19.35 ± 0.98

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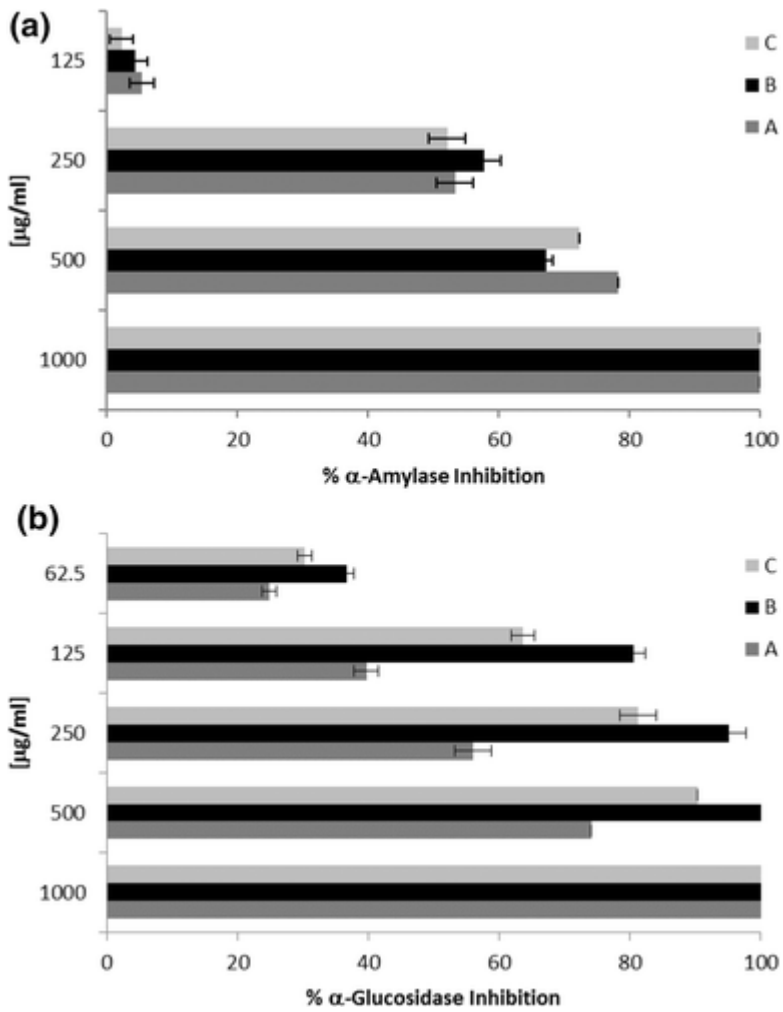
617 **Table 6 Leaf analysis of *C. × clementina***

Parameters	Zone A	Zone B	Zone C
N (%)	2.79 ± 0.20	2.24 ± 0.45	3.39 ± 0.29
P (%)	0.26 ± 0.05	0.68 ± 0.09	0.25 ± 0.06
K (%)	1.19 ± 0.20	1.45 ± 0.38	1.21 ± 0.30
Ca (%)	7.92 ± 1.78	5.24 ± 0.47	5.73 ± 0.74
Mg (%)	0.42 ± 0.09	0.49 ± 0.08	0.38 ± 0.05
Fe (mg kg ⁻¹)	134.45 ± 14.32	131.66 ± 20.03	129.80 ± 21.93
Zn (mg kg ⁻¹)	16.91 ± 4.96	15.29 ± 4.78	19.20 ± 5.70
Mn (mg kg ⁻¹)	55.65 ± 6.71	56.51 ± 9.40	48.49 ± 8.43

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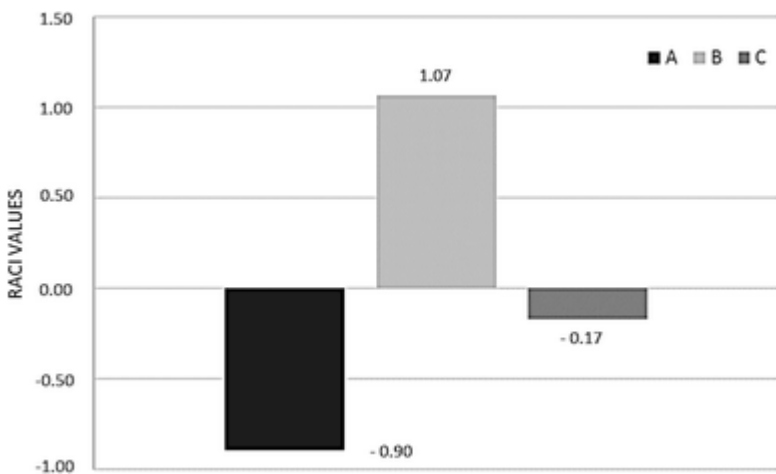


622

623 **Fig. 1.** Inhibition of carbohydrate hydrolysing enzyme by Citrus x clementina juice: a α -amylase; b

624 α -glucosidase

625

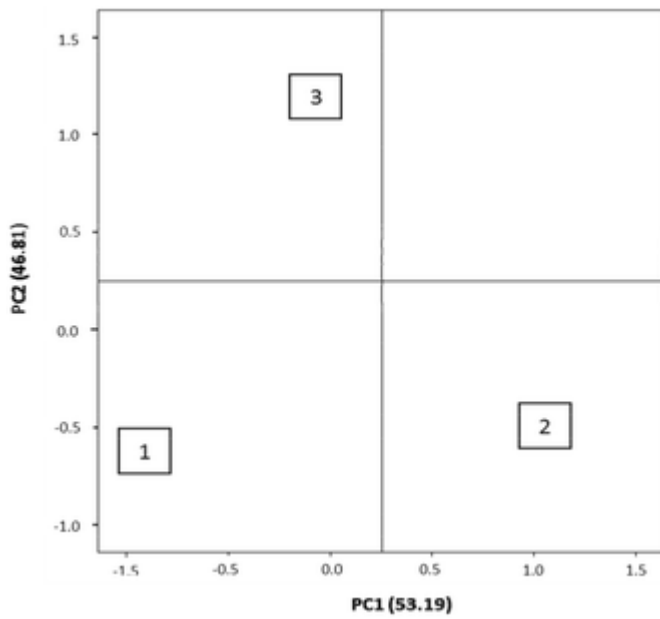


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627 **Fig. 2.** Relative antioxidant capacity index (RACI) of Citrus x clementina juices. RACI values were

628 developed from data obtained by antioxidant chemical and biological methods applied

629

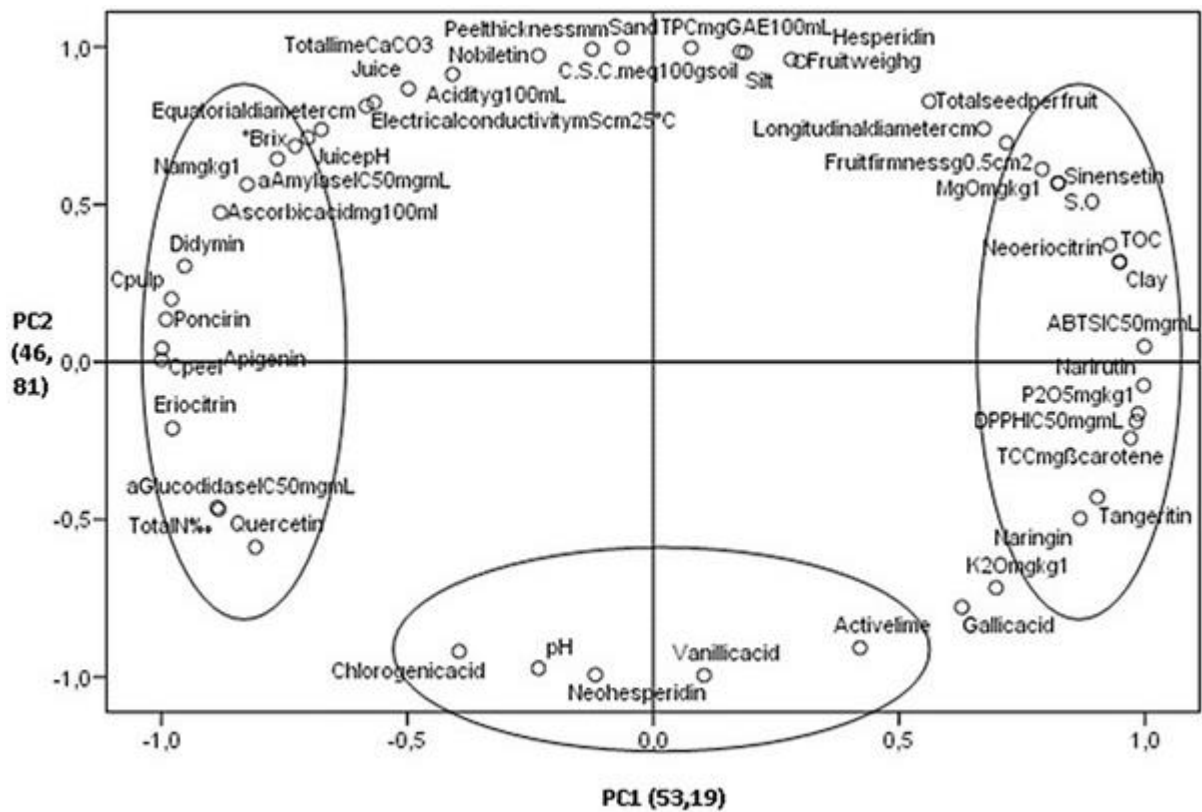


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631 Fig. 3. PCA analysis of clementine (*Citrus reticulata*) juice. Area A (1), area B (2) and area C (3):
632 score plot of PC2 against PC1.

633

634



635

636 Fig. 4. PCA loading plot (p [1] vs p [2]) for the first and second principal components