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

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## Abstract

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

**Keywords**: HPLC phenolic profle · Quality parameters · Healthy properties · PCA

## Introduction

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

Recently, the role of reactive oxygen species (ROS) in the pathogenesis of increasing number of diseases is clarified [5]. Among them, there is diabetes mellitus as a group of metabolic diseases characterized by chronic hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. This condition is associated with long-term damage, dysfunction, and failure of various organs, including eyes, kidneys, nerves, heart, and blood vessels. For the American Diabetes Association (ADA) diabetes can be classified into the following general categories: (a) Type 1 diabetes or T1DM (due to β-cell destruction, usually leading to absolute insulin deficiency), (b) Type 2 diabetes or T2DM (due to a progressive insulin secretory defect on the background of insulin resistance); (c) gestational diabetes mellitus (diabetes diagnosed in the second or third trimester of pregnancy that is not clearly overt diabetes), (d) other type. Insulin resistance is not only a key feature of T2DM, but also a consequence of exposure to inflammatory cytokines such as tumour-necrosis factor- (TNF-) that could activate c-Jun NH2-terminal kinase (JNK) [6]. Moreover, ROS may contribute to the long-term deterioration of insulin secretory capacity in the islet β-cell level since they could affect mitochondrial ATP production that is necessary for hormone secretion. Mitochondrial function also appears a critical determinant of insulin sensitivity within muscle, liver, and adipose tissue. Moreover, ROS appear important in the autoimmune destruction that characterizes type 1 diabetes, as well as in the pathophysiology of the long-term complications that characterize both classes of diabetes [7]. Between two types of diabetes, T2DM is more prevalent than type T1DM. Postprandial hyperglycaemia plays an important role in the development of T2DM so regulating plasma glucose level is crucial for delaying or preventing T2DM. One of the most common approaches to reduce or delay the intestinal absorption of glucose is by inhibiting carbohydrate hydrolysing enzymes such as α-amylase and α-glucosidase [8]. Acarbose one of the leading inhibitors of carbohydrate hydrolysing enzymes, is frequently associated with side effects such as bloating, diarrhea, flatulence, cramping, and abdominal pain [9]. During the last 10 years, several works investigated the potential role of phytochemicals as carbohydrate hydrolysing enzymes inhibitors. In this context, we have decided to screen the phenols profile, hypoglycaemic and antioxidant potential of C. × clementina juice collected in three different areas of growth. Therefore, the purpose of this study is to (a) identify the phenols HPLC profile of juice; (b) evaluate hypoglycaemic potential and the antioxidant activity of the juice (c) clarify if growth area influenced the quality parameters and phytochemical content of the juice. The findings of this research study could help C. × clementina cultivators to improve fruit quality, making it suitable for industrial products with particular reference to functional juice.

## Materials and methods

### Chemicals and reagents

All reagents used in this study were purchased from Sigma-Aldrich S.p.a. (Milan, Italy) while solvent of analytical grade was obtained from VWR International s.r.l. (Milan, Italy). Acarbose from Actinoplanes sp. was obtained from Serva (Heidelberg, Germany). Vanillic acid (≥99% HPLC grade) chlorogenic acid and gallic acid were purchased from Sigma-Aldrich Chem. Co. (Milwaukee, WI, USA). Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, didymin, neohesperidin, poncirin, quercetin, apigenin, sinensetin, nobiletin, and tangeritin were supplied by Extrasynthese (Genay–France). Acetonitrile, formic acid and water were solvent HPLC grade, obtained from Carlo Erba Reagents (Milano, Italia). Standard solutions were prepared by adding accurately weighed amount of each antioxidant compound in methanol (90:10). A calibration straight for each standard was obtained by analyzing the standard solution diluted at different concentrations. All solutions were filtered through a 0.45 μm Millipore filter (GMF, Whatman) and injected to HPLC system for retention times determination.

**Samples collection and physicochemical analysis**

The fruits of C. × clementina (Rutaceae) used in this study were collected in November 2016 in Calabria (Southern Italy), Plain of Sybaris (CS) from 20 selected farms in three different areas (zone A, B and C). The authentication was carried out at the Natural History Museum of Calabria and the Botanic Garden, University of Calabria. In zone A, the flood plain results from the Crati and Coscile rivers. The sediments, tend coarse, become finer in the less areas close to the river. The water table is found below 150 cm depth. Overall, the soils in this subzone have good productive potential, while demanding adequate water and nutrient management strategies. Zone B (Terrazzi antichi, Hill) is placed at altitudes between 50 and 80 m above sea level. This area was characterized by evolved soils, as confirmed by the red colors, with leaching and redeposition as clay pedogenetic process. The clay increase along the profile has particularly affected the hydrological and chemical properties. Zone C (Coastal plain) comprises the central part of the Plain of Sybaris, near the mouth of the river Crati, characterized by elevations between 0 and 10 m above sea level, with depressed areas behind the dunes. Reclaimed in the first half of the last century, the area currently has the water table in balance with the artificial drainage system. The substrate consists of coarse sediments with alkaline reaction soils, Franco-sandy becoming mostly sandy at about 110 cm deep. The low water retention capacity and low cation exchange capacity make it advisable to adopt adequate water management strategies and nutrient. C. × clementina requires a mild climate, as constant as possible during the growing season, and is particularly sensitive to temperature changes, especially those caused by cold winds that dry the twigs. In the plain, the summer temperatures reach a maximum value of 40 °C, while winter temperatures are around 4 °C with differences of 2–4 °C above the hill. To create appropriate temperature conditions during the winter period, plants are covered with dark green plastic nets.

Fruits were randomly harvested from 30 healthy homogeneous trees per grove. Twenty-five fruits for each area of growth (A, B, and C) were collected and examined for integrity and absence of insect and dust contamination. Physical characteristics of the fruits such as fruit weight (g), equatorial diameter (cm), longitudinal diameter (cm), fruit firmness (g/0.5 cm2), peel thickness (mm), total seeds per fruit and amount of extracted juice (%) were determined. Samples were freeze-dried and stored at −20 °C until analysis. The total nitrogen content, moisture content, ash content, fat content, crude fiber content, total carbohydrates, minerals, and energy values were evaluated [10].

C. × clementina fruits 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 higher saturation of color or chroma (C*). Total soluble solids (TSS) were determined using a digital refractometer PR-201α (Atago, Tokyo, Japan), previously calibrated at 20 °C and the results are 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 (IFU): 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 of anhydrous and hydrate citric acid. Ascorbic acid was determined using the International Federation of Fruit Juice producers test (IFU): a potentiometric titration of the acidity of the juice, with a solution of 2,6-dichloroindophenol [11]. All determinations above described were made in triplicate.

**HPLC analysis of juice**

C. × clementina juice was pre-treated with centrifuge (4000 rpm for 20 min), then filtered by using membrane filter (0.45 μm). Separation of phytochemicals was made by using a HPLC apparatus equipped with Phenomenex C18 column (150 mm × 3 mm) according to the official methodologies of the International Federation of Fruit Juice producers [15]. Two buffers were used as solvent system (A: Acetonitrile/Water/phosphoric acid (70:26:4) and B: Potassium Dihydrogen Phosphate at pH 3.5). 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. The chromatogram was monitored at λ = 287 nm. Identification of compounds was performed by comparing their retention time with those of standards, and confirmed with characteristic spectra using the photodiode array detector.

**Carbohydrate hydrolysing enzymes inhibition study**

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

%Inhibition=100−([Maltose]test[Maltose]control×100)±S.D.

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

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

$\%\text{Inhibition}=100-([\text{Glucose}]\text{test}[\text{Glucose}]\text{control}\times 100)\pm\text{S.D.}$

**Radical scavenging activity**

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

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

ABTS assay was applied by using the previously published methodology with slight modifications [14]. ABTS radical cation (ABTS+) was mixed with potassium persulphate and left in the dark for 12 h before use. The ABTS+ solution was diluted with ethanol to an absorbance of 0.70 ± 0.05 at λ = 734 nm by using a Perkin Elmer Lambda 40 UV/VIS spectrophotometer. A mixture of juice and diluted ABTS+ solution was prepared and after 6 min the absorbance was measured at 734 nm. The scavenging ability of the juice was calculated according to the following equation: ABTS scavenging activity (%) = [(A 0 − A)/A 0] × 100 where A 0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

DPPH radical scavenging assay was previously described by Loizzo et al. [14]. An ethanol solution of DPPH radical (DPPH·) at concentration of 1.0 × 10−4 M was mixed with juice. The reaction mixtures left in the dark for 30 min. The absorbance was measured at λ = 517 nm against blank without DPPH. The DPPH radical scavenging activity (%) was calculated according the following equation:

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

Relative antioxidant capacity index (RACI) calculation

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

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

**Soil and leaf analysis**

Soils were sampled in March, before the annual application of fertilizers, using a manual drill. In each grove, four soil specimens were collected at depths of 0–30 cm and 30–60 cm and mixed to form a single sample. Total $CaCO_3$ (%) was determined using a calcimeter [16]. Organic matter (%) was assessed using the Walkley–Black method, available P (μg/g) was determined by the Olsen method while Kjeldahl method was used for total N (‰) [16, 17]. Atomic absorption spectrophotometry was applied to determine exchangeable Ca, K, Na and Mg cations (μg/g) [16]. The pH was also measured (PH211 pH meter, HANNA Instruments). The leaves were picked in October when the level of elements was stable and before fertilizers were applied. The leaves collected for analysis were 5–7 months old. Foliar analysis was performed on 30 leaves from the index trees picked from non-fruit bearing terminal shoots of the year's spring flush [18]. Determination of total nitrogen was made as previously reported while inductively spectrometer plasma (ICP) technique was used for macro and micro-elements [19].

**Statistical analysis**

All experiments were carried out in triplicate. Data are expressed as mean ± standard deviation (S.D.). The concentration giving 50% inhibition (IC50) 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 control. To achieve the objectives set, the data obtained were processed using statistical procedures that tended to highlight any significant relationship of nutrients in the leaves and in the physico-chemical parameters of soil with the phenolics identified in C. × clementina juice. Studies of the Pearson's correlation coefficient (r) and linear regression, assessment of repeatability, calculation of average and relative standard deviation was performed using Microsoft Excel 2010 software. Moreover, differences between zone A, B and C were underlined. Similarities between clementine juices and differences between three geographical areas (A, B, C) were studied. One-way ANOVA (Tukey's test) and Principal Component Analysis (PCA) were applied by SPSS software for Windows, version 15.0 (Chicago, IL, USA).

**Results and discussion**

*Fruits quality parameters*

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

*C.×clementina juice analysis*

Each investigated sample give a percentage of juice ranging from 45.61 to 49.60% for zone B and C, respectively, (Table 2). The C. × clementina juice quality parameters, including pH, total soluble solids, total acidity, ascorbic acid content, and color were investigated. The values of total acidity ranged from 0.35 to 0.88 g citric acid/100 mL for juice obtained from fruits collected in zone B and C, respectively. The highest total soluble solids (11.63 °Brix) were detected with juice obtained from fruits collected in coastal plain. A pH ranging from 3.50 to 3.76 was measured. Moreover, juice from fruits collected in coastal plain is rich in ascorbic acid (66.25 mg/L). No significant differences were evidenced in chroma value (C*). Samples are rich also in phenols and carotenoids with a total phenol content ranging from 29.74 to 44.20 mg GAE/100 mL for zone A and C, respectively, and from 42.89 to 75.45 mg β-carotene/100 mL for A and B, respectively. Lower values of vitamin C were detected by Bermejo et al. [20] that screened 15 mandarin cultivars. Among them, Arrufatina, Loretina and Fina displayed the highest values of total vitamin C, followed by the hybrid Ellendale.

Previously, Al-Mouei and Choumane [21] determined the quality parameters of twelve Citrus varieties namely common mandarin, mandalina, clementine, Nova, Carvalhal, Dancy, Klimntard, Fortune, Ortanique, Minneola, Ponkan and Satsuma growing in Syria. Among all investigated varieties group, Ortanique had the highest juice content (56.1%) while common mandarin had the lowest content (37%). The TSS in mandarin group ranging from 9.5 to 13.9 °Brix. Our results on pH measurement are in line with those reported by the authors that found a pH value of 3.5 for Clementine while Fortune variety showed the lowest value 2.67. Great variability on total acidity was found also for mandarin group with Minneola that contained high TA value (1.62 g citric acid/100 mL) while Nova has lowest acidity value (0.49 g citric acid/100 mL).

Juices from Cadoux, Monreal, St. Martin, Merme, Cheylard, and Rocamora clementine cultivar and one mandarin fruit cultivar were investigated for quality parameters and antioxidant potential [22]. The TA of investigated juices ranged from 4.61 to 7.47 g of citric acid/L for clementine cv. Merme and mandarin, respectively, with a pH varied between 3.91 and 3.68 for the same cultivars, respectively. Ascorbic acid content was lowest in clementine St Martin cv. (35.98 mg/100 mL). Mineral analysis showed that all investigated cultivars are rich in potassium.

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

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

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

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

**Carbohydrate hydrolysing enzyme activities**

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

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

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

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

**Radical scavenging activity**

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

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

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

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

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

**Soil and leaves parameters**

Analysis of soil in the different areas of collections evidenced that almost all physical and chemical parameters were significantly different (Table 5). The soil in the plain at sea level was characterized by a high sand content (64.22%), and by lower P2O5 and K2O. In contrast, the soil in the area in the flood plain presented a lower content of total lime (6.89%) in comparison with the plain at sea level (8.96%). Leaf analysis was used to determine the nutrient status of the tree and to understand the nutritional requirements of C. × clementina tree. As evidenced in Table 6 all investigated leaf shows an optimal content micro and macro-nutrients. The nitrogen content was particularly high in zone B (3.39%), probably caused by the higher intake of nitrogen fertilizer. Significant differences were noted for Ca with higher levels in the flood plain area. Significantly different was also data regarding P since it is three times higher in hill (zone B) respect zone A and C.

**Principal component analysis (PCA)**

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

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

## Conclusion

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

## Abbreviations

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

***ADA:*** American Diabetes Association

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

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

***PCA:*** Principal component analysis

***ROS:*** Reactive oxygen species

***T1DM:*** Type 1 diabetes

***T2DM:*** Type 2 diabetes

***TNF-α:*** Tumour-necrosis factor-α

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**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 |

| 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 |
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| Phosphorus | 19 | 21 | 22 |
| Potassium | 177 | 179 | 185 |
| Calcium | 30 | 32 | 32 |
| Magnesium | 14 | 10 | 14 |

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 cm2) | 300.56 ± 1 3.98 | 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 |

**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 |
|---|---|---|---|
| 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 |
| Neoeriocitrin | 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 |

**Table 4 Hypoglycaemic activity and radical scavenging activity of *C.* × *clementina* juice ($IC_{50}$ 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-Glucodidase | 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*** |

**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 |
| P2O5 (mg kg−1) | 128.07 ± 9.89 | 188.07 ± 11.64 | 145.09 ± 10.24 |
| K2O (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 (CaCO3 %) | 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 |

**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 |

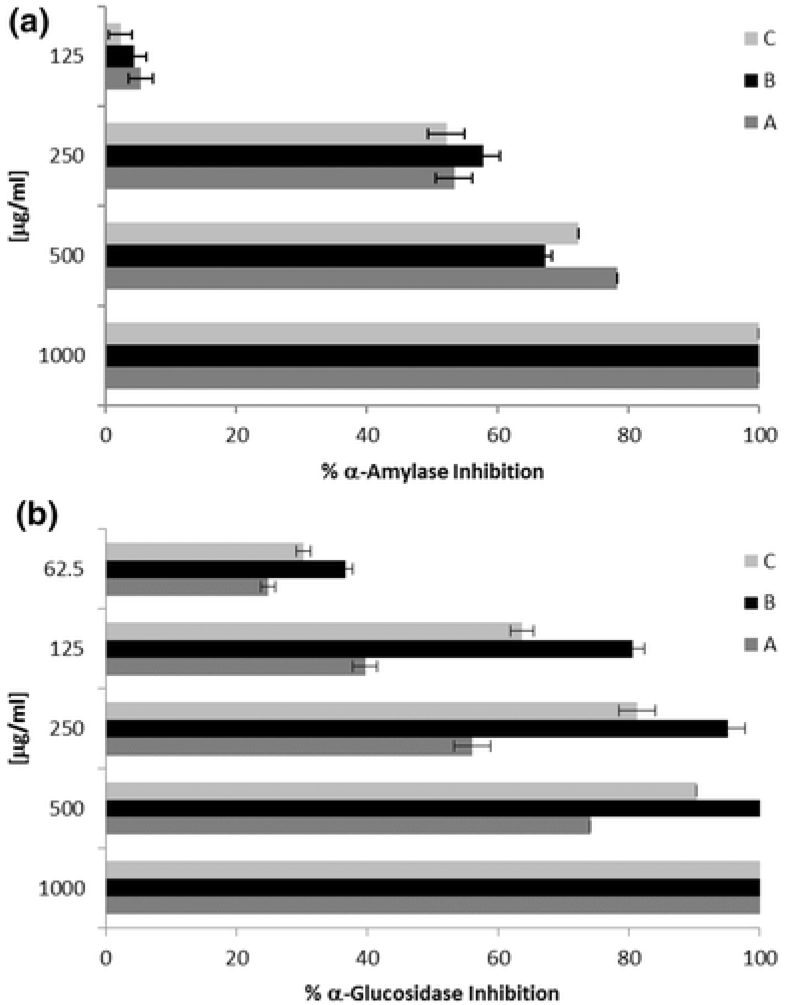


**Fig. 1.** Inhibition of carbohydrate hydrolysing enzyme by Citrus × clementina juice: a α-amylase; b α-glucosidase

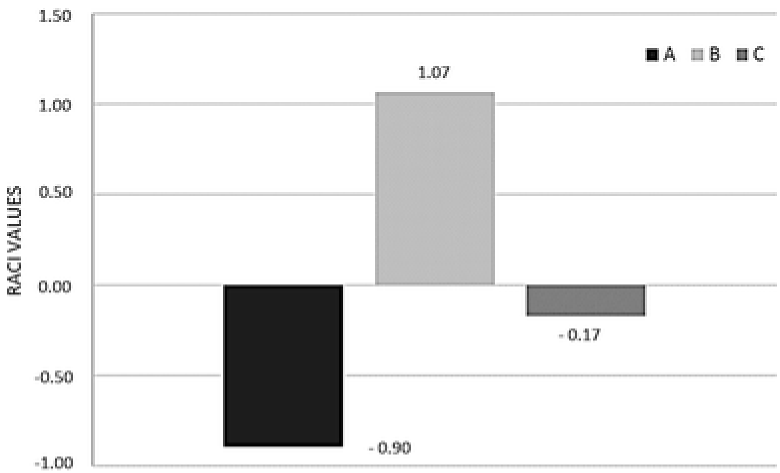


**Fig. 2.** Relative antioxidant capacity index (RACI) of Citrus × clementina juices. RACI values were developed from data obtained by antioxidant chemical and biological methods applied

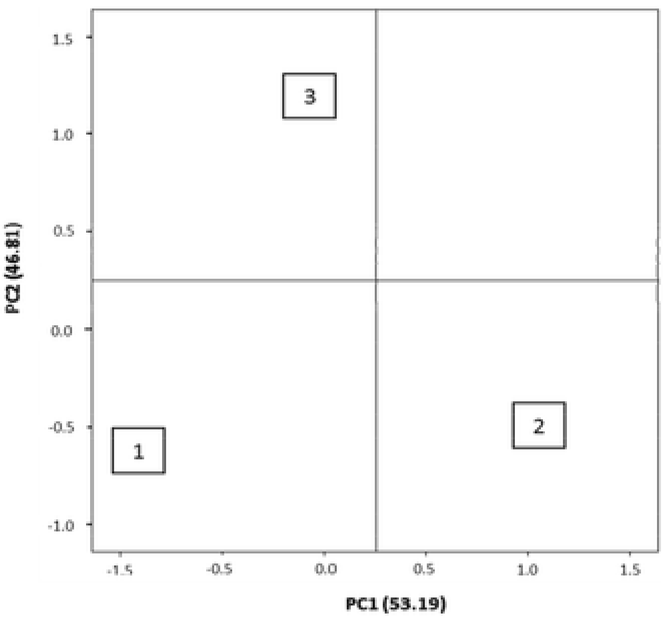


Fig. 3. PCA analysis of clementine (Citrus reticulate) juice. Area A (1), area B (2) and area C (3): score plot of PC2 against PC1.

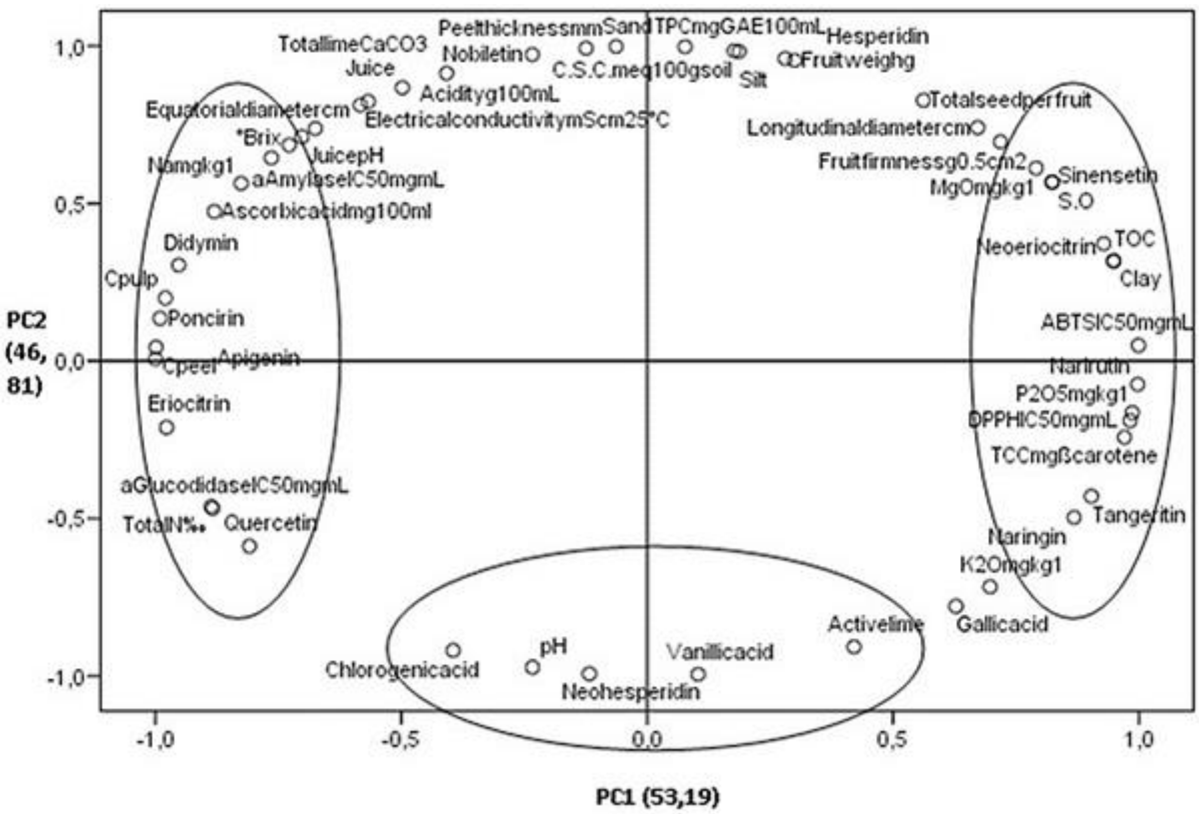


**Fig. 4**. PCA loading plot (p [1] vs p [2]) for the first and second principal components