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Preliminary Study on Bioactive Compounds of *Citrus × myrtifolia Rafinesque* (Chinotto) to Its Potential Application in Food Industry

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ABSTRACT

The present study investigated for the first time some physical quality attributes of unripe Citrus × myrtifolia Rafinesque which is the ingredient of the popular soft drink Chinotto. Samples for analysis were processed to better reproduce the crude materials used for industrial chinotto extract, discarding part of the juice. Fruit bioactive compounds such as ascorbic acid, carotenoids, chlorophylls, flavonoids and antioxidant capacity were estimated. An important nutritional aspect arose from the data presented was the high concentration of flavonoids (780 *mg*/100*g FW*) *and vitamin C* (42 *mg*/100*g FW*). *A good antioxidant capacity* (5872 *μM Trolox equivalents*/100*g FW*) *was estimated by oxygen radical absorbing capacity* (*ORAC*). *This matrix could be considered as a good nutraceutical source, giving new opportunity to citrus industry.*

***Keywords*:** *Ascorbic Acid*, *Antioxidants*, *Chinotto*, *Citrus*, *Flavonoids*, *ORAC*

1. Introduction

The *Citrus × myrtifolia Raf.* is a citrus fruit of the genus Citrus. Native of southern China, its origin has not been exactly ascertained; probably it is mutation of sour orange that eventually evolved into the species known today [1]. The unripe fruits look like small green aromatic tangerines, while mature fruits are bigger and orange painted. The flesh is bitter and sour and divided into 8 - 10 segments. The plant was cultivated for centuries in France and Italy [1], especially in Liguria, Calabria and Sicily where the fruits are used in sweets for candies and jams as well as flavoring syrups, soft drinks and spirits. In Italy the plant has given its name to a very popular Italian drink Chinotto, flavored with *Citrus × myrtifolia* extract. The chinotto is set up as a classic soda, but no producer has been revealed details about its preparation. Its ingredients are regulated at the legislative level by a Decree of President of the Republic (DPR n.719 of 1958 May 19, Article 5 and subsequently amends) which specifies that the soft drinks sold under the name of a not juice fruit, including cedar and chinotto, should be prepared with substances derived from the fruit or the plant. The *Citrus × myrtifolia* extract for chinotto drink is a aqueous-alcoholic solution made up from infusion of softly pressed unripe fruits with partially discarding of the juice; it was then flavored with spices such as rhubarb, gentian, cinchona, cinnamon, cloves, sweet orange, thyme, tamarind. The first Chinotto soda was produced in 1932 by San Pellegrino®.

Despite the growing distribution of this popular soft drink, the few literature papers about the *Citrus × myrtifolia* [2,3] are related only to the juice and no informations are available about bioactive compounds in the whole fruit. The have of interest in the Italian citrus industry for chinotto production could be increased by studies on its composition.

The possible beneficial effects of fruit consumption are due to micronutrients such as vitamins and to functional food ingredients and antioxidant nutraceuticals (phytochemicals) [4]. Phytochemicals can be defined as substances found in edible fruits and vegetables that, daily ingested, may exhibit a potential for modulating human metabolism to the prevention of chronic and degenerative diseases. An increased consumption of fruit

and vegetables, typical Mediterranean diet foods, may protect against degenerative pathologies, such as cancer and atherosclerosis [5]. Over the past decades, a large number of studies have been carried out with the aim of identifying the bioactive components present in citrus fruits, in an attempt to gain a deeper understanding of the correlation between diet, health benefits and reduced risk of diseases [6-8]. Among the phytochemicals, flavonoids are widely contained in Citrus fruits, especially in Sicilian cultivars, but are also good source of other bioactive components such as carotenoids and vitamin C [9].

The aim of the present work was to characterize for the first time unripe *Citrus* × *myrtifolia* raw materials used for beverage industrial purposes. The main bioactive compounds such as carotenoids, ascorbic acid, flavonoids and chlorophylls were quantified. Furthermore, the radical scavenging activity was screened using ORAC method.

2. Materials and Methods

2.1. Fruits

Fruits used in this study were grown in Castiglione di Sicilia (Catania, Italy). Immature *Citrus* × *myrtifolia Raf.* (chinotto) fruits were collected with the degree of fruit maturity determined from the surface color, mean diameter, total soluble solids (TSS), titratable acidity (TA) and pH. Unripe fruits picked on 2010 October were green, had a mean diameter of 2.5 (±0.3) cm and a mean weight of 27 g (±2). The peel and the pulp represented the 39% and the 55% of the fresh weigh (FW) respectively, while the remaining weigh were constituted by seeds. The TSS was 8.4 Brix, TA of 0.83% citric acid and pH 3.87; the evaluated ratio (TSS/TA) for maturity requirements was of 10.1. Samples for analysis were prepared from 25 fruits and processed to better reproduce the crude materials used for industrial chinotto extract; the fruits were slightly squeezed to discard part of the juice and reduced in small pieces with Turbo Homogenizer HMHF (PBI International). The samples were stored at –20˚C until needed for the study.

2.2. Extraction and Analysis of Flavonoids

Two grams of sample was extracted for 2 h with 10 mL of 80% methanol containing 1% hydrochloric acid at room temperature on an orbital shaker. The mixture was centrifuged at 1000 rpm for 15 min and the supernatant decanted. The pellets were extracted under identical conditions. Supernatants were combined and used for total flavonoid assay. The flavonoid content was determined in all of the samples as indicated by Association of the Industry of the Juices and Nectars (AIJN) according to the colorimetric Davis method [10]. Ten milliliters of diethylene glycol were added to 0.2 ml of water/methanolic (50:50, v:v) extract and mixed. Thereafter 0.2 ml of approximately 4 N sodium hydroxide is added and the increase in color is read at 420 nm after 5 minutes with Perkin-Elmer LAMBDA 35 UV/VIS spectrophotometer. The observed color increases were compared with a standard curve prepared from the ACS grade pure naringin (Sigma Aldrich, Italy). The values were expressed as mg naringin/100g FW (fresh weight).

2.3. Extraction and Analysis of Chlorophylls

The extraction of pigments were performed in subdued light in 90% acetone and the content of chlorophylls a and b were calculated in according with formulas proposed by Jeffrey and Humphrey in 1975 [11] from the absorption spectra recorded at 645 and 663 nm:

$$\text{chlorophyll a} = 11.93 \times A663 - 1.93 \times A645$$

$$\text{chlorophyll b} = 20.36 \times A645 - 5.50 \times A663$$

The values were expressed as μg/g FW.

2.4. Extraction, Saponification and Analysis of Carotenoids

A 25 g amount of sample were accurately weighed and transferred to a 250 ml amber Erlenmeyer flask. Then, 0.5 g of ascorbic acid (Sigma Aldrich, Italy), 50 ml of absolute ethanol and 10 ml of 60% potassium hydroxide (Carlo Erba, Italy) solution were added, under a stream of nitrogen. Saponification was performed overnight with slow constant stirring at room temperature. Then, the saponified mixture was transferred to a 250 ml amber separatory funnel, rinsed with 30 ml water and extracted five times (shaking for 2 min) with three fractions containing 50 ml of n-hexane and two fractions containing 25 ml of n-hexane. The combined n-hexane extract was washed with 50 ml fractions of water, which were added with some drops of phenolphthalein, until the aqueous layer appeared colorless. A 1 g amount of BHT butilidrossitoluene (BHT) (Sigma Aldrich, Italy) was added as the antioxidant and the mixture was then passed through a Whatman No. 1 filter containing 20 g of anhydrous sodium sulfate (Carlo Erba, Italy) and was collected into a 250 ml amber volumetric flask. The extract was concentrated by rotatory evaporation at 40˚C. Finally, the evaporated residue was reconstituted with n-hexane and the absorbance at 470 nm of extracts was determined. The pigment concentration was expressed in μg β-carotene per g FW using an external calibration prepared from the pure β-carotene (Sigma Aldrich, Italy). The corresponding content of provitamin A was calculated in term of Retinol Equivalent (R.E.) per gram of

FW (1 μg R.E. = 6 μg *β*-carotene).

2.5. Total and Reduced Ascorbic acid Content

The used procedure is based on the method of Kampfenkel *et al* [12] for the spectrophotometric determination of ascorbic acid as ascorbate (AsA) and dehydroascorbate (DAsA). The assay is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and the spectrophotometric detection of complexed Fe^{2+} with 2,2-dipyridyl (Sigma Aldrich, Italy). DAsA was reduced to AsA by preincubation of the sample with dithiothreitol (Sigma Aldrich, Italy). Subsequentely the excess dithiothreitol was removed with N-ethylmaleimide (Sigma Aldrich, Italy), and the total AsA was determined from the difference of total AsA and AsA (without pretreatment with dithiothreitol). The Vitamin C content was expressed as mg/100g FW, by comparison with a standard curve of AsA (Sigma Aldrich, Italy).

2.6. Ascorbate Peroxidase (APX) Assay

The APX activity was assayed following Wang *et al.* [13]. APX extraction was performed with a 50 mM potassium-phosphate buffer (pH 7.0) in presence of 1 mM ascorbic acid to avoid the enzyme inactivation during extraction. APX activity was evaluated on a reaction mixture made up of 0.5 ml enzyme extract in 50 mM potassium-phosphate buffer (pH 6.6), 1 mM ascorbic acid, 4 mM H_2O_2, 0.4 mM Na_2EDTA, following the extinction rate of ascorbic acid due to its oxidation by H_2O_2. The reaction was started with the addition of H_2O_2 and the ascorbic acid degradation was followed monitoring the decrease of absorbance at 290 nm at 25˚C. Ascorbate peroxidase specific activity was expressed in $U \cdot mg^{-1}$ of enzymatic proteins.

2.7. Enzymatic Protein Determination

The enzymatic proteins content was determined according to the method of Bradford [14], using Coomassie® Brilliant Blue R (Sigma Aldrich, Italy) which shows, in the free form, a maximum absorbance peak at 465 nm. The reagent, which binds mainly to the residues of arginine and, to a less extent, to lysine, histidine, tyrosine, tryptophan and phenylalanine of the enzyme, shows a maximum absorbance peak at 595 nm. One ml Coomassie® was added to variable aliquots of enzyme. After 15 minutes the absorbance at 595 nm was read and the protein amounts were calculated using a calibration curve obtained with bovine serum albumine (Sigma Aldrich, Italy) at concentrations ranging from 2 to 10 μg.

2.8. Determination of the Total Antioxidant Capacity

Aliquots of sample were extracted with phosphate buffer 75 mM/l (pH 7.4) and after sonicated for 60 s. The extract was centrifuged at 10000 rpm for 10 min at 4˚C. The total antioxidant capacity was assayed on the supernatant according to ORAC (Oxygen Radical Absorbance Capacity)-fluorescein assay [15]. Trolox standards (10 - 100 μM/l), fluorescein (7.0 μM/l), and 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (63 mM/l) solutions (Sigma Aldrich, Italy) were prepared prior to use in phosphate buffer (75 mM/l, pH 7.4). Chinotto samples and different concentrations of trolox (10 - 100 μM/l) were pipetted into 96 well of assay plates and the total volume was adjusted to 40 μl by the addition of buffer. Further, 200 μl of working fluorescein solution was added. The plate was then allowed to equilibrate by incubating for 3 min at 37˚C in the PerkinElmer VICTOR3 V Multilabel Counter 1420. Reaction was initiated by the addition of 20 μl of AAPH solution using the microplate reader's injector. The fluorescence was then monitored kinetically with data taken every three min. The plate was top read at excitation and emission wavelengths of 485 and 535 nm, respectively at 37˚C and at 3 min intervals for 60 min. The standard curve was obtained by plotting trolox concentrations against the average net area under the curve (AUC) of the three measurements for each concentration. The net AUC corresponding to a sample was calculated by subtracting the AUC of the blank. A standard curve was generated from the net AUC of the trolox standards and used to assign trolox equivalence values to the samples. Final ORAC values were calculated using the regression equation between trolox concentration and the net AUC and are expressed as micromol trolox equivalents per 100 gram of FW.

2.9. Statistical Analysis

To verify the statistical significance of all parameters the values of means and standard deviation (SD) were calculated. Where it was appropriate, the data by two-way ANOVA were tested. The $P_{value} < 0.05$ were adopted as statistically significant. All data are means of five measurements. Average amounts of bioactive compounds and antioxidant activity in *Citrus* × *myrtifolia Raf.* FW basis were reported in **Table 1**.

3. Results and Discussion

Flavanone glycosides are the most abundant phenolic compounds present in citrus fruit, but significant concentrations of other flavonoids such as methoxylated flavones and flavonols have also been found [16,17]. Among them, literature data available for chinotto juice demonstrated that flavanone 7-O-neohesperidoside components neoeriocitrin, naringin, and neohesperidin pre-

dominate in *Citrus* × *myrtifolia* [2,3]. Therefore the total flavonoids content determined by Davis method was reported as the main flavonoids naringin [2], referring to standard curve. The total flavonoids found in *Citrus x myrtifolia* extract were 780 mg/100 g FW (**Table 1**). It's well known that phenolic contents were usually higher in peels [18] and in immature fruits [3], thus it is not entirely surprising that this flavonoids amount was more and more high to what reported for chinotto juice; literature data reported values of total juice flavonoids ranging from 57.4 mg/L [3] and about 2000 mg/L [2].

Carotenoids in citrus fruits are localized in plastids present in both the flavedo (containing about 70% of the total in fruit) and in the vesicles that contain the juice. When the fruit is immature their color is masked by chlorophylls, with the progress of ripening yellow appears in various shades from pale yellow to deep orange due to variations in type and quantity of different carotenoids [19]. Due to the presence of chlorophylls, immature fruits are capable of photosynthesis but cannot make significant contribution to own nutrition. There is a rapid synthesis of carotenoids in the chromoplast during ripening, which is accompanied by a simultaneous loss of chlorophylls [19]; in many citrus there is no further synthesis of colored carotenoids during ripening after all chlorophylls had disappeared [20]. The analyzed samples contained in fact a high amount of total chlorophylls (41.4 μg/g FW) (**Table 1**).

Table 1. Average amounts of bioactive compounds and antioxidant activity in *Citrus* × *myrtifolia Raf.* on fresh weigh (FW) basis.

Parameter	*Mean Value*[a]
Total flavonoids (mg/100g)[b]	780 (13)
Total chlorophylls (μg/g)	41.4 (1.8)
chlorophyll a (μg/g)	*36.8* (*1.5*)
chlorophyll b (μg/g)	*4.7* (*0.2*)
Total carotenoids (μg/g)[c]	1.7 (0.1)
Provitamin A (μg R.E. [d]/g)	0.28 (0.01)
Ascorbic acid (mg/100g)	41.8 (3.1)
ascorbate (mg/100g)	*35.0* (*1.3*)
dehydroascorbate (mg/100g)	*6.8* (*1.8*)
APX [e] (U/mg enzymatic proteins)	12.88 (0.05)
ORAC[f] total antioxidant activity (μM T.E.[g]/100 g)	5872 (351)

[a]mean value of five determination, standard deviation in parenthesis ($P_{value} < 0.05$); [b]expressed as naringin; [c] expressed as *β*-carotene; [d]retinol equivalents; [e]ascorbate peroxidase activity; [f]Oxygen Radical Absorbance Capacity; [g]trolox equivalents.

Studies about effect of ethylene application on chlorophylls in Navelate fruit harvested [21] demonstrated that a decrement of chlorophylls occurred during maturation from about 115 μg/g to 84 or 7 μg/g depending on air or ethylene post-harvest. Chlorophylls in citrus consist mainly of two pigments: chlorophyll a and chlorophyll b; chlorophyll c, d and chlorophyll e are not reported in citrus and are mainly present in algae and certain sea weed [19]. Chlorophyll a and b were present in the sample in the ratio 7:1. Chlorophyll a/b ratio is an indicator of the functional pigment equipment and light adaptation of the photosynthetic apparatus; chlorophyll b is found exclusively in the pigment antenna system, whereas chlorophyll a is present in the reaction centers of photosystems I and II and in the pigment antenna [22].

On the other hands, total carotenoids were present only in the alkaline saponified extract to demonstrate that at this maturation stage carotenoids are present bonded form. The saponification of the extract of carotenoids has been traditionally an important step in the protocol for the determination of these pigments. Xanthophylls are usually esterified in fruits, the degree of esterification depending on the number of hydroxyl groups in the molecule [7]. Thus, monol carotenoids like *β*-cryptoxanthin may be found either free or as monoesters; diol carotenoids, such as lutein or zeaxanthin, may be free or esterified by one or two fatty acids, and so forth. Furthermore, the saponification reaction leads to the elimination of the chlorophylls present in the samples. Chinotto total carotenoids content of 1.7 μg/g FW (**Table 1**) is quite low; the published total carotenoid content in the peels of unripe oranges is about ten times higher [21,23]. Carotenoids with an unsubstituted b-ring with an 11-carbon polyene chain such as *β*-cryptoxanthin and *β*-carotene are nutritionally important because of the vitamin A activity, since they are converted to retinal by mammals. This role is of particular importance, especially in developing countries where the dietary deficiency of vitamin A can lead to blindness and premature childhood mortality [24]. The provitamin A activity estimated from total carotenoids content corresponded to 0.28 μg R.E./g (**Table 1**). Aside from the nutritional relevance of some carotenoids owing to their vitamin A activity, these pigments are increasingly drawing the interest of researchers as they may be somehow implicated in the prevention and/or protection against major human diseases [7].

Citrus fruits are rich in vitamin C; the abundance of citrus in the Mediterranean diet may provide the main dietary source for natural this vitamin [19]. Daily intake

of 5 mg is sufficient to prevent symptoms of scurvy in an adult. The intake of 30 - 60 mg is estimated to be required for full grown adults. The limiting step for vitamin C absorption in humans is transcellular active transport across the intestinal wall where AsA may be oxidized to dehydroascorbic acid (DAsA), which is easily transported across the cell membrane and immediately reduced back to AsA by two major pathways [25]. Quantitative analyses of the chinotto sample are in agreement with literature references for citrus fruits [19] showing a content of 41.8 mg/100g, which 84% biologically active reduced form (AsA) (**Table 1**). AsA bioavailability in the presence of flavonoids has yielded controversial results. Whereas flavonoids seem to inhibit intestinal absorption of AsA, some studies have shown that AsA in citrus extract was more available than synthetic ascorbic acid alone [25]. DAsA is reported to possess equivalent biological activity to AsA, so recent studies often consider the vitamin C activity in the diet as the sum of AsA plus DAsA [25]. Oxidative damage has many pathological implications in human health, and AsA may play a central role in maintaining the metabolic antioxidant response.

In whole fruit the enzymatic system of oxidation of AsA is intact, while when the fruit is processed some losses of vitamin C may occur [19]. Because steady-state levels of e reactive oxygen species (ROS) depend on the balance between ROS -producing and -scavenging reactions, we measured levels of both AsA and DAsA together the activity of ascorbate peroxidase (APX); the latter plays a major role in scavenging H_2O_2 in plants, because can oxidize AsA [19]. The value estimated in fresh sample was of 12.88 U/mg enzymatic proteins (**Table 1**).

The measurement of the antioxidant capacity of food products is a matter of growing interest because it may provide a variety of information, such as resistance to oxidation, quantitative contribution of antioxidant substances, or the antioxidant activity that they may present inside the organism when ingested [26]. The studied chinotto showed high antioxidant effect (5872 μM Trolox equivalents/100g FW), measured with ORAC method (**Table 1**). The antioxidant activity industrially pro- cessed chinotto fruit was determined for the first time in this study and therefore no data are available in the literature to compare with our results. On the basis of the of edible portion of fruit, it was demonstrated that strawberry had the highest ORAC activity (about 1500 μM Trolox equivalents/100g FW), followed by plum, orange, red grape, kiwi fruit, pink grapefruit, white grape, banana, apple, tomato, pear, and melon (about 100 μM Trolox equivalents/100g FW) [27].

The elevate antioxidant activity may be mostly attributed to their high content of polyphenols. The correlation between total phenol contents and antioxidant activity has been widely studied in different foodstuffs such as fruit and vegetables [28-30]. As reported, antioxidant activity of fruits and vegetables significantly increases with the presence of high concentration of total polyphenols content [31-33]. It has been demonstrated flavonoids have 2 - 6 times the antioxidant activity of common antioxidants, such as ascorbic acid [34].

The absence of correlation between ORAC and ascorbic acid values was found by Rapisarda *et al.* [35] on different citrus hybrids. The same results were obtained by Wang *et al.* [36] for orange juice and Prior *et al.* [34] for Vaccinium species. Shahidi and Marian [37] reported that differences in antioxidant activities of fruits could be due to their different structures from phenolic acids and flavonoid compounds as well as their derivatives. Results of Rababah *et al.* [33] showed that the concentrations of antioxidants in strawberry, peach, and apple mixed with 0.1% ascorbic acid were very similar to those without addition of ascorbic acid.

4. Conclusions

Our results indicated that the *Citrus* × *myrtifolia* raw materials used for beverage industrial purposes is a good source of phytochemicals, mainly vitamin C and flavonoids, and may therefore provide health benefits to consumers. The overall amounts of bioactive compounds in whole fruit are in fact higher than in the juice. Nowadays these aspects are considered to be highly valuable for the commercial valorization of chinotto as a citrus with high potential as nutraceutical source.

The studied matrix contain a group of natural antioxidants that have not only a high antioxidant activity, but also a good antioxidant quality that could enrich lower density lipoproteins, thereby protecting them from oxidation and preventing development of atherosclerosis and other diseases. The supplementation of natural antioxidants through a balanced diet containing enough fruits could be much more effective and economical than the use of individual antioxidants for protecting of the body against various oxidative stresses. Neither chlorophylls nor carotenoids can be synthesized by animal tissues, though animal cells can chemically modify them for assimilation. Thus, these molecules must be obtained from food.

Moreover recent research has demonstrated the possibility of recovering anthocyanins, flavanones and hydroxycinnamic acids from blood orange juice or citrus byproducts and using this extract as an antioxidant ingredient for dietary supplements [38,39]. Carotenoid and

chlorophyll molecules could be also extracted and used as natural colorants and antioxidants [40]. This approach on chinotto matrix could represent a new and important strategy in the citrus industry.

Further works are in progress in our laboratory to elucidate the identity of compounds responsible for nutraceutical source.

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