UNIVERSITÀ DELLA CALABRIA

Dipartimento di Farmacia e Scienza della Salute e della Nutrizione

Scuola di Dottorato

Science and Technique Bernardino Telesio

Indirizzo

Organic Materials of Pharmacological Interest

CICLO XXVIII

Doctoral Thesis

Bioactive compounds and properties of Chinotto (*Citrus Myrtfolia* Raf.) at different ripening stages

Settore Scientifico Disciplinare CHIM10

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To my parents,

and my beloved husband

ABSTRACTS

Citrus myrtifolia Raf. (chinotto) is a citrus fruit which originates from a mutation of *Citrus aurantium* (sour orange). This fruit has a small size and looks like a ping-pong. In Italy, The plant is cultivated in Calabria, Sicily and Liguria. The fruit of chinotto is widely used in food industry. Concentrated chinotto juice is a key ingredient in the production of soft drinks and liqueurs, whereas the whole fruit is used in the confectionery industry. However, despite its industrial applications, chinotto is among the least studied citrus fruit, with only a few reports present in the literature.

Here, we studied bioactive compounds contained in all parts of chinotto fruit. We also investigated the changes of those bioactive compounds during maturation and different area cultivation. First, the oil content from the seeds were analyzed, in order to assess the fatty acid profiles. After that, the methanolic extracts and DMF extracts of the defatted seeds and pulps were analyzed for their total phenolic contents (TPC) and antioxidant capacities. The results showed that all those extracts had the ability to scavenge both DPPH and ABTS radicals. TPC value is highly dependent on the level of fruit maturity. Moreover, we also isolated the pectin and β-glucan from chinotto seeds and pulps, and characterized them using FT-IR, in order to provide the valuable information about the new alternative sources of pectin and β-glucan.

Essential oils were extracted from the peels. The effects of maturation on the composition in volatile compounds of those essential oils were evaluated. After that, the potential antioxidant and anti-inflammatory properties of these fractions were tested. Our finding showed that essential oil obtained from semiripe chinotto peels effectively modulates inflammation in vitro and could, therefore, represent a potential attractive source of bioactive compounds for food, cosmetic, or pharmaceutical applications.

Key words: Chinotto, fatty acid profile, antioxidant, total phenolic content, pectin, β-glucan, essential oil, anti-inflamatory

ACKNOWLEDGEMENTS

This thesis has been written as a part of my three year PhD at Department of Pharmacy, Health and Nutritional Sciences in the University of Calabria, Italy, between May 2013 and February 2016.

It is a pleasure to thank the many people who made this thesis possible. Firstly, I would like to express my special appreciation and thanks to my supervisor Dr. Alessia Fazio, she has been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. I could not have imagined having a better supervisor for my PhD study. Her invaluable help of constructive comments and suggestions throughout the thesis works have contributed to the success of my PhD program.

My sincere thanks also goes to Dr. Pierluigi Plastina for his advice and friendly assistance with various problems during my PhD. I greatly appreciate his time in explaining many things before I faced my final exam.

I cordially thank Prof. Galileo Violini and Dott. Gianpiero Barbuto, for their support and help towards my scholarship affairs. Next, I would like to thank Prof. Bartolo Gabriele, coordinator of OMPI curriculum and Prof. Roberto Bartolino, director of 'Bernadino Telesio' Doctoral school of science and technology, UNICAL.

My sincere appreciation also extends to all my colleagues in laboratory of food chemistry who have provided assistance at various occasions. Unfortunately, it is not possible to list all of them in this limited space.

I would also like to thank all my friends in Centro Residenziale, especially Lisa, Yuges, Dhanya, Narjes, Rihab, Wafa, Zahira, Rahmat,Youssef and others for their help, kindness and moral support during my study. Thanks for the friendship and memories.

Last but definitely not the leastI would like to thank my family members. Words cannot express how grateful I am to my mother and father, for all of the sacrifices that you've made on my behalf. Your prayer for me was what sustained me thus far. I would also like to thank my beloved husband, Rhesa Adriansyah. Thank you for supporting me for everything, and especially I can't thank you enough for encouraging me throughout this experience. You are the apple of

my eye. To my mother-in-law, father-in-law, my brother, sisters-in-law and brother-in-law who have been so supportive for my overseas studies. Thank you and I love you all so much!

Praise to God for His help and guidance that I finally able to complete this dissertation.

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CHAPTER 1

INTRODUCTION

1.1 *Citrus myrtifolia* Raf.

Citrus myrtifolia Raf., commonly known as chinotto or myrtle-leaved orange, is a species belonging to the *Rutaceae* family, subfamily *Aurantioideae* and genus *Citrus*, which originates from a mutation of *C. Aurantium* (sour orange) [1]. Native of southern China, its origin has not been exactly ascertained. The plant was cultivated for centuries in France and Italy. In Italy the production of chinotto fruits are concentrated in the southern regions, for its mild climate and the type of terrain, with Sicily in the first row, followed by Calabria and the Ligurian Coast (Savona). This plant is a woody plant varies from shrub to small tree one meter high, with rounded crown and compact branches with leaves of dark green color, small and similar in size to those of the myrtle; hence the Latin name Myrtifolia. The flowers are small, white and very fragrant. The fruits are in clusters, a deep orange color and the weight no more than 50-60 grams, with the size as a ping-pong. Immature fruits are green oblate spheroids with a diameter of about 2-3 cm, whereas ripe fruits are orange oblate spheroids with a diameter of about 5-6 cm. The flesh is bitter and sour and divided into 8–10 segments. The peel is tightly adherent to the pulp [2] .

Fig.1.1 Chinotto fruits

Although in many countries it is grown only for ornamental purposes, its sour-tasting fruits have a significant impact on the food industry. The smaller green ones (2-3 cm diameter) are used in the candy industry or in the preparation of jam, whereas the juice of ripe fruits is an essential flavor component of syrups, soft drinks and aperitifs and, above all else, the primary ingredient of the ‘Chinotto’ Italian soft drink. In light of the growing distribution of this popular soft drink, which is also beginning to appear in several other countries (America, Australia), it is surprising

that chinotto fruit is among the least studied citrus fruit, with only a few reports present in the literature [3-5].

1.2 History of Chinotto

Chinotto (*Citrus Myrtifolia* Raf.) is a nativeplant from southern China. In the sixteenth century, the Portuguese came to Goa, India, where the tree cultivated. The Indians considered the plant was not local but native to the areas of South-East Asia and China.

The interest was due to the fact that this fruit was extracted as an important perfume essence and also as a source of vitamin for the sailor because of the presence of many antioxidants made it suitable for the storage on the sailing ships on long journeys. It was imported to Europe because those purposes mentioned. People in Turkey, Syria, and the Black Sea area used chinotto also as a perfume essence. After that, people started to consume squeezed chinotto juice with a lot of sugar as a beverage or as an ingredient of candy.

In the eighteenth century, the cultivation of chinotto had spread to Mediterranean. It was difficult to plant chinotto tree in that area,800 cultivations were greatly reduced because of the difficulty of cultivation (slow growth, poor harvests in case of drought, plant death in case of frost, the discovery of their durable for sea traveller. Currently, the cultivation of chinotto has completely disappeared from the Iberian Peninsula, from Provence, from North Africa and the East from Turkey and Syria. However, in France the trees of chinotto exist only in the area of Nice (which until 1860 was part of Italy).

According to history document, it seems that in Italy chinotto was imported by a navigator of Savona in 1500. At that time chinotto was used to make candy or jelly. Over the centuries it spreads across the western coast a real "industry chinotto" that knows its greatest success in the last century. At the end of the Second World War the cultivation of chinotto was present only in Liguria (from Ventimiglia to Nervi) and Sicily. The fruits "turned" ("turning" is the operation that allows the removal of the thin layer of peel), "boiled", "tanned" and placed in brine were sold in wooden tubs: departed from the port of Savona to Marseille for the confectionery sector.

Nowadays, the industriesof chinotto are growing only in the province of Savona (Albenga) with a high cultivation in the area from Pietra Ligure to Finale Ligure. In Sicily it is found only in the area of Taormina. At present the Liguria together with Georgia remains growing chinotto with the seeds which are imported from Asia.

1.3 Habitat of Chinotto plants

Chinotto, like all other citrus fruits belongs to the family of Rutaceae, suitable to be planted in subtropical climates and particularly sensitive to the extremely cold. These plants are very resistant to excess water and various diseases. It is very suitable for growing in pots. It is grown mainly in the warmer regions of Italy for its mild climate and the type of soil in both conducive to plant growth (Liguria, Calabria and Sicily).

1.4 Bioactive compound in Chinotto

It is known how citrus fruits are rich in different phytochemicals, which all contribute to determine a high nutraceutical potential: several studies have shown that these compounds possessbiological properties such as antioxidant, anti-inflammatory andanalgesic effects, anti-viral and anti-bacterial activities, up to antithrombotic, neuroprotective and anti-tumoral properties [6].

Several studies reported that almost all parts of chinotto fruit contain many bioactive compounds that may provide health benefits to human body. Chinotto raw materials used for beverage industrial purposes is a good source of phytochemicals, mainly vitamin C and flavonoids. These bioactive compounds in whole fruit of chinotto are in fact higher than in the juice [7]. Barreca *et al.* identified that chinotto contained significant amount of flavonoids and furocoumarins in their peel, carpel membranes, leaves and seeds. The main phenolic components of chinotto fruits belong to the flavanone class, including narigin and neohesperidin as the major flavonoid components. The remaining flavanones contained in small quantity, such as neoeriocitrin, melitidin, brutieridin, eriocitrin, and narirutin. The other compounds that also present in chinotto in lower amount than flavanone groups, namely rhoifolin,vicenin-2, lucenin-2,polymethoxyflavone, bergapten, epoxybergamott, neodiosmin, sinensetin, tetramethoxyfavone, nobiletin, heptamethxyflavone and tengeretin [8,9,10].

Nowadays,all those aspects above are considered to be highly valuable for the commercial valorization of chinotto as a citrus with high potential as nutraceutical source. However, this study only focused on naringin and neohesperidin contents in chinotto pulp and their changes during maturation because based on the previous studies naringin and neohesperidin were the major compounds in chinotto.

1.4.1 Naringin

Naringin (with the molecular formula $C_{27}H_{32}O_{14}$ and a molecular weight of 580.4 g/mol) is a flavanone-7-O-glycoside between the flavanone naringenin and the disaccharide neohesperidose. Two rhamnose units are attached to its aglycon portion, naringenin, at the 7-carbon position. Naringin contents in various citrus species such as Chinotto fruit, and it presents in high amount in the whole part of chinotto and also in their leaves [8]. Naringin is responsible for the fruit's bitter taste.

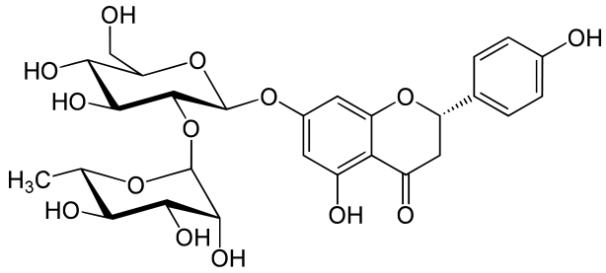

Fig.1.2 Chemical structure of naringin

Naringin are strong antioxidants and have an abilty to scavenge free radicals and prevent lipid peroxidation. Both superoxide and hydroxyl radicals are scavenged by this flavonoids in vitro [11,12].Naringin is moderately soluble in water. The gut microflora breaks down naringin to its aglycon naringenin in the intestine; it is then absorbed from the gut [13]. Although the average daily human intake of naringin or flavonoids is not known, the total intake of polyphenols was suggested as ~1 g/d [14]. Chun et al. [15] estimated flavonoid intake by combining the USDA flavonoids database and 24-h dietary recall in NHANES 1999–2002 data. The daily mean intake of flavonoids was found from tea (157 mg), citrus fruit juices (8 mg), wine (4 mg), and citrus fruits (3 mg).

In metabolic syndrome, obesity, and related cardiovascular complications, naringin influences AMPK-, PPARα–, and CPT-1–mediated fat utilization and preserves mitochondrial function. Moreover, naringin also prevents the TNF-α–mediated inflammatory process and tissue damage in liver and vasculature. Naringin supplementation lowered elevated plasma lipid concentrations in high-fat-diet–fed rats and decreased plasma lipids and cholesterol in high-cholesterol-diet–fed rats. The cholesterol-lowering effect of naringin was observed in LDL receptor (LDLR) knockout mice. Hepatic 3-hydroxy- 3-methyl CoA (HMG-CoA) reductase activity was significantly reduced in the naringin-supplemented (0.02 g/100 g) group, whereas cholesterol

acyl transferase (ACAT) activity was unaffected in Ldlr knockout mice [16]. In a clinical trial, naringin supplementation (400 mg/capsul/d) reduced plasma total- and LDL-cholesterol concentrations [11]. Moreover, naringin significantly increased the production of NO metabolites in urine and improved the acetylcholinemediated endothelium function using thoracic aortic ring preparations by NO production [17]. Effect ofNaringin on Cardiac Toxicity and Hypertrophy is reducing lipid peroxidation, improved antioxidant enzymes, and decreased inflammatory cell and fibrosis in hearts of isoproterenol-treated rats [18]. Other study showed that naringin (30 mg/kg) and vitamin C (50 mg/kg) cotreatment ameliorated streptozotocininduced diabetes in rats by improving insulin concentration and prevented oxidative stress. It also improved insulin concentration and pancreatic architecture in db/db mice at a supplementation dose of 0.2 g/kg of diet [19].

1.4.2 Neohesperidin

Neohesperidin is a flavonoid which belong to flavanone glycoside group . Its aglycone form is called hesperetin [20]. Citrus fruits are rich sources of neohesperidin; the peels of oranges, lemons, and grapefruit contain bitter taste of neohesperidin. alkaline hydrogenation of neohesperidin can produce Neohesperidin dihydrochalcone (NHDC) compound, which is an intensive sweetener.

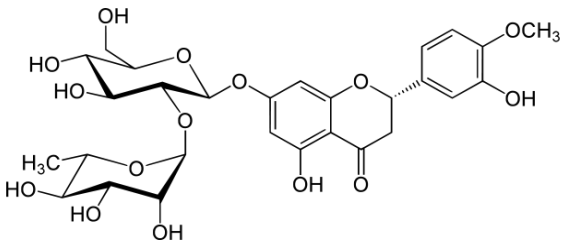

Fig.1.3 Chemical structure of Neohesperidin

NHDC showed remarkable radical scavenging activity against stable radical and reactive oxygen species (ROS). NHDC was the most potent inhibitor of H_2O_2 and HOCl. NHDC showed HOCl scavenging activity of 93.5% and H_2O_2 scavenging property of 73.5% which was more than those of all the tested compounds including ascorbic acid and BHT. Moreover, NHDC could inhibit protein degradation, plasmid DNA strand cleavage and HIT-T15, HUVEC cell death from HOCl attack while mannitol, BHT, and ascorbic acid could not protect them effectively. Hence,

NHDC is a potent antioxidant, especially it is evaluated as a novel HOCl scavenger. The intake of NHDC-containing food might contribute positive in allergic patients. There are many advantages on NHDC for drug development because of its characteristics of intense sweetener, low toxicity, and potent antioxidant activity [21]

1.5 Fatty Acid

Fatty acid is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated. Most naturally occurring fatty acids have a chain of an even number of carbon atoms, from 12 to 28 [22]. Fatty acids are usually derived from triglycerides or phospholipids. When they are not attached to other molecules, they are known as "free" fatty acids.Fatty acid chains differ by length, often categorized as short to very long:

1. Short-chain fatty acids (SCFA) are fatty acids with aliphatic tails of fewer than six carbons (e.g. butyric acid).
2. Medium-chain fatty acids (MCFA) are fatty acids with aliphatic tails of 6–12 carbons, which can form medium-chain triglycerides
3. Long-chain fatty acids (LCFA) are fatty acids with aliphatic tails 13 to 21 carbons.
4. Very long chain fatty acids (VLCFA) are fatty acids with aliphatic tails longer than 22 carbons [23, 24].

Fatty acids are usually produced by the hydrolysis of triglycerides, with the removal of glycerol. Phospholipids represent another source. Some fatty acids are produced synthetically by hydrocarboxylation of alkenes.

Fatty acids that are required by the human body but cannot be made in sufficient quantity from other substrates, and therefore must be obtained from food, are called essential fatty acids. There are two series of essential fatty acids: one has a double bond three carbon atoms removed from the methyl end; the other has a double bond six carbon atoms removed from the methyl end. Humans lack the ability to introduce double bonds in fatty acids beyond carbons 9 and 10, as counted from the carboxylic acid side [25]. Two essential fatty acids are linoleic acid (LA) and alpha-linolenic acid (ALA). They are widely distributed in plant oils. The human body has a limited ability to convert ALA into the longer-chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can also be obtained from fish.

Fatty acid consist of 2 types, first fatty acids that have carbon–carbon double bonds are known as unsaturated and second the fatty acids without double bonds are known as saturated. They differ in length as well.

1.5.1 Saturated Fatty Acid

Saturated fatty acids have no double bonds, Unbranched, straight-chain molecules. Thus, saturated fatty acids are saturated with hydrogen (since double bonds reduce the number of hydrogens on each carbon). Because saturated fatty acids have only single bonds, each carbon atom within the chain has 2 hydrogen atoms (except for the omega carbon at the end that has 3 hydrogens). The short-chain, low molecular weight fatty acids (<14:0) are triglyceride constituents only in fat and oil of milk, coconut and palmseed. In the free form or esterified with low molecular weight alcohols, they occur in nature only in small amounts, particularly in plant foods and in foods processed with the aid of microorganisms, in which they are aroma substances [26]. Fatty acids belong to saturated fatty acid are behenic acid (C:22), myristic acid (C:14), palmitic acid (C:16), stearic acid (C18:0), arachidic acid (C:20), etc.

Palmitic acid (hexadecanoic acid) (Fig. 1.4) is one of the most common fatty acids found in food. Palmitic acid contained in palm oil, ruminant milk fat, butter, cheese, fruit coat fats, seedfats and in fatty meat [27].

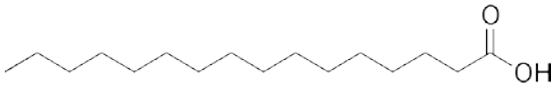

Fig. 1.4 Structure of Palmitic acid

Myristic acid (acid tetradecanoic) (Fig. 1.5), whose name comes from Myristica fragrans, a tropical tree of nutmeg seeds, which have a very high concentrations, up to 70-80% of the lipid fraction. Myristic acid also present in tropical oils, especially as palm and coconut, and animal fats (cheese and meat). The myristic acid is used to produce soaps and cosmetics, since its salts (sodium and potassium) have foaming properties. An ester, isopropyl myristrate is used in topical preparations for promoting the cutaneous absorption.

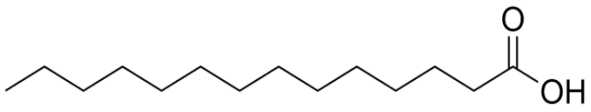

Fig. 1.5 Stucture of myristic acid

Stearic acid (octadecanoic acid) (Fig. 1.6) is not responsible for heart disease because the body is rapidly desaturated to oleic acid with the intervention of Stearoyl-CoA desaturase [28]. Stearic acid can be found in foods high fat foods, especially animal source food, and also in vegetables, especially in cocoa butter and shea butter.

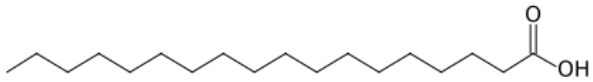

Fig. 1.6 Stucture of stearic acid

Behenic acid (acid docosanoic) (Fig. 1.7) is a carboxylic acid, the saturated fatty acid with formula $C_{21}H_{43}COOH$. In appearance, it consists of white to cream color crystals or powder with a melting point of 80 °C and boiling point of 306 °C. Behenic acid is extracted from the seeds of the Ben-oil tree (*Moringa oleifera*). It is so named from the Persianmonth *Bahman*, when the roots of this tree were harvested. Behenic acid is also present in some other oils and oil-bearing plants, including rapeseed (canola) and peanut oil and skins.

Commercially, behenic acid is often used to give hair conditioners and moisturizers their smoothing properties. It is also used in lubricating oils, and as a solvent evaporation retarder in paint removers. Its amide is used as an anti-foaming agent in detergents, floor polishes and dripless candles. Reduction of behenic acid yields behenyl alcohol [29].

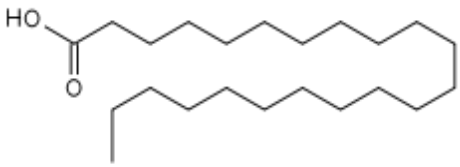

Fig. 1.7 Structure of behenic acid

Arachidic acid (fig 1.8), also called eicosanoic acid, is the saturated fatty acid with a 20-carbon chain. It is as a minor constituent of peanut oil (1.1%–1.7%), corn oil (3%) and cocoa butter (1%). Its name derives from the Latin arachi, peanut. It can be formed by the hydrogenation of arachidonic acid.Reduction of arachidic acid yields arachidyl alcohol.Arachidic acid is used for the production of detergents, photographic materials and lubricants [30].

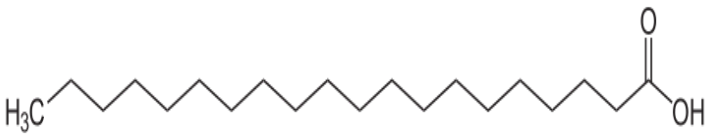

Fig. 1.8 Structure of Arachidic acid

1.5.2 Unsaturated Fatty Acid

The unsaturated fatty acids, which dominate lipids, have one or more double bonds between carbon atoms. (Pairs of carbon atoms connected by double bonds can be saturated by adding hydrogen atoms to them, converting the double bonds to single bonds. Therefore, the double bonds are called unsaturated). In most natural fats the double bonds of unsaturated fatty acids occur in the cis configuration. In milk fat a considerable proportion is in the trans configuration. These trans bonds result from microbial action in the rumen where polyunsaturated fatty acids of the feed are partially hydrogenated [27, 31].

Oleic acid (C18: 1) (Fig. 1.9) is a monounsaturated fatty acid belonging to the family of the ω-9. The oleic acid contain in olive oil with the percentages around 60-80%, it presents in esterified form (triglycerides).

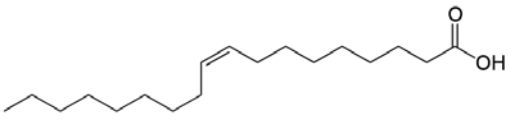

Fig. 1.9 Structure of Oleic acid

Linoleic acid is an essential fatty acid with 18 carbon atoms. It is a key precursor of some endogenous bioregulators such as prostaglandins, which play a very important role in inflammatory processes, and thromboxanes, involved in blood clotting.

Linoleic acid can be linked to the lowering of total cholesterol, by acting on sensitization to lipoprotein receptor of the liver, with the disadvantage (absent for the ω-9) to slightly reduce the high density lipoproteins (HDL or "good cholesterol"). For some specialists, the non-selective cholesterol lowering action of linoleic acid can be defined negligible, since complications related to hypercholesterolemia relating especially to the alteration of the relationship between the

lipoproteins (LDL / HDL). In percentage terms, the linoleic acid lowers LDL much more than those of HDL, which in general will tend to stabilize.

Linoleic acid (Fig. 1.10) is contained mainly in sunflower seeds, wheat germ, in sesame, walnuts, soybean, corn, and olive oil.

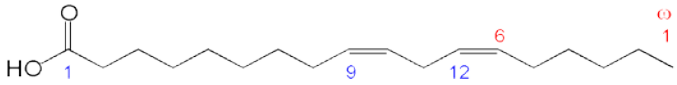

Fig. 1.10 Structure of linoleic acid

α-Linolenic acid (ALA) (Fig. 1.11) is a carboxylic acid with an 18-carbon chain and three cis double bonds. The first double bond is located at the third carbon from the methyl end of the fatty acid chain. Thus, α-linolenic acid is a kind of omega-3 fatty acid which is found in plants and an isomer of gamma-linolenic acid (GLA), a polyunsaturated n−6 (omega-6) fatty acid. It is found in flaxseed oil, and in canola, soy, and walnut oils. These vegetable oils should be obtained by cold pressing, possibly with the addition of vitamin E, used strictly for raw salad dressings, and stored in dark glass containers, to protect from light and heat sources. α-linolenic acid is similar to the omega-3 fatty acids that are in fish oil, called eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Human body can change α-linolenic acid acid into EPA and DHA. However, some researchers suggest that less than 1% of ALA is converted to physiologically effective levels of EPA and DHA.

The main functions of α-linolenic acid acid are antiplatelet, anti-thrombotic and vasoprotective. Moreover, omega-3 fatty acids, especially EPA and DHA, have been shown to reduce inflammation and may help prevent chronic diseases, such as heart disease and arthritis. They may also be important for brain health and development, as well as normal growth and development. Fish oil containing EPA and DHA may help treat heart disease, prevent heart attack and stroke, and slightly reduce high blood pressure [27,31,32].

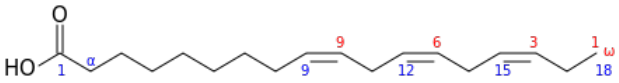

Fig. 1.11 Structure of α-linolenic acid

Palmitoleic acid (fig 1.12), or (9*Z*)-hexadec-9-enoic acid, is an omega-7 monounsaturated fatty acid with the formula $CH_3(CH_2)_5CH=CH(CH_2)_7COOH$ that is a common constituent of the glycerides of human adipose tissue. It is present in all tissues but, in general, found in higher concentrations in the liver. It is biosynthesized from palmitic acid by the action of the enzyme delta-9 desaturase. A beneficial fatty acid, it has been shown to increase insulin sensitivity by suppressing inflammation, as well as inhibit the destruction of insulin-secreting pancreatic beta cells [33].

Palmitoleic acid can be abbreviated as $16{:}1\Delta^9$. Dietary sources of palmitoleic acid include a variety of animal oils, vegetable oils, and marine oils. Macadamia oil (*Macadamia integrifolia*) and sea buckthorn oil (*Hippophae rhamnoides*) are botanical sources with high concentrations, containing 17% and 19% min to 29% max of palmitoleic acid, respectively [34].

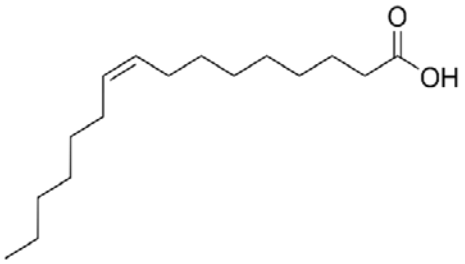

Fig. 1.12 Structure of Palmitoleic acid

1.6 Pectin

Pectin is a polysaccharide mixture with a complicated structure containing at least 65% of galacturonic acid (GalA). Three structural elements are involved in the make-up of a pectin molecule: a homogalacturonan consisting of (1 → 4) linked α-D-GalA, a galacturonan with differently arranged side chains (building blocks: apiose, fucose, arabinose, xylose), and a rhamnogalacturonan with a backbone consisting of the disaccharide units [→ 4)-α-D-GalA-(1 → 2)-α-L-Rha-(1 →] and with its rhamnose residues linked by arabinan and galactan chains [26].

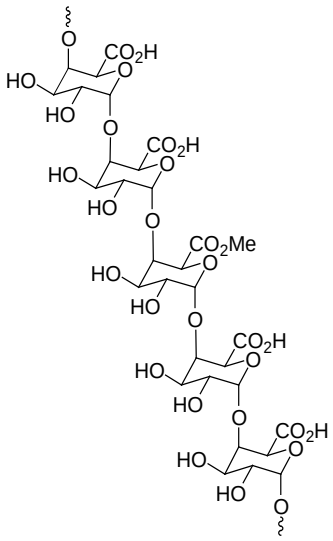

Fig. 1.13 Chemical structure of pectin

Pectin occurs commonly in most of the plant tissues as a cementing substance in the middle lamella and as a thickening on the cell wall, thereby providing vegetable tissue with consistency and mechanical resistance. However, during postharvest ripening of fruits, pectins typically undergo solubilization and depolymerisation which causes softening of the fruit texture. The number of sources that may be used for the commercial manufacture of pectins is very limited [35]. The main sources of commercially acceptable pectins are from peels of citrus fruits and from apple pomace. It is 20–40% of the dry matter content in citrus fruit peel and 10–20% in apple pomace [36,37]. Alternative sources include sugarbeet waste from sugar manufacturing, sunflower heads (seeds used for edible oil), and mango waste [38].

Pectin is a part of natural human diet, as the literature reports, the daily intake of pectin from fruit and vegetables can be estimated to be around 5 g (the consumption of approximately 500 g fruit and vegetable per day is estimated). Pectin acts as a soluble dietary fiber [39]. Consumption of pectin has been shown to reduce blood cholesterol levels. The mechanism appears to be increase of viscosity in the intestinal tract, leading to reduce absorption of cholesterol from bile or food [39, 40]. In the large intestine and colon, microorganisms degrade pectin and liberate short-chain fatty acids that have favourable influence on health (also known as prebiotic effect). Pectin has been used potentially as a carrier for drug delivery to the gastrointestinal tract, such as matrix tablets, gel beads, film-coated dose form [40].

In food industry, pectin is used as a gelling agent in a wide range of fruit-based products, such as jams, marmalades, jellies, fruit preparations for yoghurts and desserts and fruit filling for bakery products. Pectin can be used to improve the mouth-feel and the pulp stability in juice based drinks and as a stabiliser in soured milk beverages, yoghurts and ice creams. Pectin also reduces syneresis in jams and marmalades and increases the gel strength of low calorie jams [26,41,42].

The percentage of carboxyl groups esterified with methanol is the degree of esterification (DE) or degree of methylation (DM). Degree of methylation in pectin is an important factor characterizing pectin chains. DM of pectin molecules can influence gel formation in food industry as thickening agents [37]. There can be a wide range of DMs dependent on species, tissue, and ripening [31,43, 44]. It has been observed that the DM of pectins changes during fruit ripening [45]. The degree of methyl-esterification (DM) has a strong impact on the functional properties of pectins and these are categorized as high-ester or low-ester with DM > 50% and DM < 50%, respectively [46]. High methoxyl pectin can form a gel under acidic conditions in the presence of high sugar concentrations or a similar co-solute at pH < 3.5, whereas low methoxyl pectin forms gels only in the presence of a polyvalent salt, usually the calcium ion (Ca^{2+}) at a very wide range of pH (between 2.6 and 7.0) [47].

1.7 β-glucans

β-glucans are indigestible polysaccharides occurring naturally in various organic sources such as corn grains, yeasts, bacteria, algae, fungi and higher plants such as fruit, corn grains, oats, barley, rye. β-glucans from different sources have different linkage types, branching manners and MW [26]. They are important components of the fibres containing unbranched polysaccharides consisting of β-d-glucopyranose units linked through (1→4) and (1→3) glycosidic bonds in cereals, (1→6) glycosidic bonds in fungal sources, and (1→3) and (1→ 6) glycosidic bonds in baker's yeast [48,49].

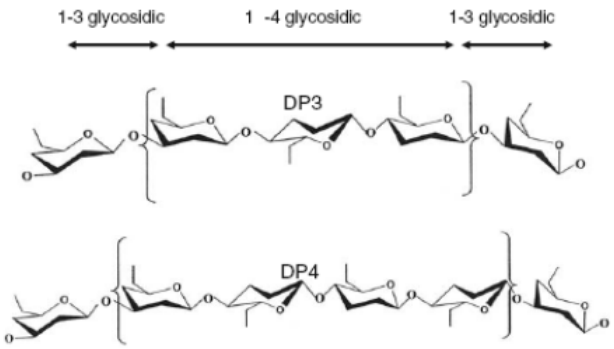

Fig. 1.14 Chemical structure of β-glucan

The structure has an impact on the water solubility of β-glucans. Extensive research has been done into the structure and properties of water solubleβ-glucans in contrast to water-insolubleβ-glucans. Generally, no sharp distinction exists between the soluble and insoluble fractions and the ratio is highly dependent on the extraction conditions of the soluble fibre. Glucans are usually concentrated in the internal aleurone and subaleurone endosperm cells walls [50].

The health functions of β-glucan have attracted much attention in recent years. Besides being a source of dietary fiber, it is linked with certain biomedical effects such as host defense potentiator (HDP), antitumor, anti-infective, immunostimulator, reduce total cholesterol and low-density lipoprotein (LDL) levels of hypercholesterolemia and has immunomodulatory activities.

When taken orally in foods, β-glucans reduce postprandial serum glucose levels and the insulin response, that is, they moderate the glycemic response, in both normal and diabetic human subjects [31]. The main attribute of β-glucans that makes them beneficial is the fact that they can form very high viscose solutions, therefore, increase intraluminal viscosity. Further biological effects ofβ-glucans, which may be prospectively utilised in the clinical practice, can reside in the stimulative action on the haematopoiesis of the bone marrow, and also in the radioactive and antimutagenic effects (scavengers of free radicals) [48].

In food Industries, β-glucans had an important technological role in processed foods, where they can be used for the elaboration of products with high dietary fiber content as non-caloric thickening and stabilizing agents, as an aid in the production of cheese and ice-cream, as a fat substitute in dairyproducts and as a gel-forming component [51].

1.8 Essential oil

An essential oil is defined internationally as the product obtained by hydrodistillation, steamdistillation or dry distillation or by a suitable mechanical process without heating (for Citrus fruits) of a plant or some parts of it [52]. They are aromatic oily liquids, volatile, characterized by a strong odour, rarely coloured, and generally with a lower density than that of water. They can be synthesized by all plant organs (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and root) and therefore extracted from these parts, where they are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes [53]. Essential oils only represent a small fraction of plant's composition; nevertheless they confer the characteristics by which aromatic plants are used in the food, cosmetic and pharmaceutical industries.

The aroma of each oil results from the combination of the aromas of all components, and even minor oil constituents may have major organoleptic roles to play [54]. In addition to the extraction techniques reported above there are other ones that may be used for extracting the volatile fraction, nevertheless this cannot be called an "essential oil" in those cases. Suchtechniques include: vacuum distillation, solvent extraction combined off-line with distillation, simultaneous distillation-extraction (SDE), supercritical fluid extraction (SFE), and microwaveassisted extraction and hydrodistillation (MAE and MA-HD), static (S-HS), dynamic (D-HS) and high concentration capacity headspace (HCC-HS) sampling [52]. These authors in a synthetic way explain how all of these techniques operate.

Essential oils have a complex composition, containing from a dozen to several hundredcomponents. The great majority of components identified in essential oils includes terpenes (oxygenated or not), with monoterpenes and sesquiterpenes prevailing. Nevertheless, allyl- and propenylphenols (phenylpropanoids) are also important components of some essential oils [55].

Capillary gas chromatography is the technique of choice for the analysis of essential oils due to thevolatility and polarity of essential oil components, combining two different-polarity stationary phases. Identification of oil components is generally performed by chromatographic data (Kováts indices, linear retention indices, relative retention time, retention time locking) and/or by spectral data, mainly by mass spectrometry (GC-MS).

In Nature, essential oils play an important role in the attraction of insects to promote the dispersion of pollens and seeds or to repel other ones. In addition, essential oils may also act as antibacterials, antivirals, antifungals, insecticides, herbicides, or have feeding deterrent effects against herbivores by reducing their appetite for such plants. Essential oils have also an important role in allelopathic communication between plants [56]. The detection of some of these biological properties needed for the survival of plants has also been the base for searching similar properties for the combat of several microorganisms responsible for some infectious diseases in humans and animals. This search intends to respond to the increasing resistance of pathogenic microbes to antibiotics.

The essential oils were also reported have ability to combat bacteria from the respiratory tract, anti-*Helicobacter pylori*, anti-*Mycoplasma pneumoniae*; essential oils against DNA virus: HSV1 (herpes simplex virus), HSV-2, NDV (Newcastle disease); or RNA virus: SARS-Cov (severe acute respiratory syndrome-associated coronavirus), and Junin virus. In addition, essential oils have also revealed to be effective on the inhibition of growth and reduction in numbers of the more serious foodborne pathogens such as *Salmonella* spp., *E.coli*O157:H7 and *Listeria monocytogenes* [57].

The antioxidant activity of essential oils is another biological property of great interest because they may preserve foods from the toxic effects of oxidants [58]. Moreover, essential oils being also able of scavenging free radicals may play an important role in some disease prevention such as braindysfunction, cancer, heart disease and immune system decline. Increasing evidence has suggested that these diseases may result from cellular damage caused by free radicals [59].

There is also evidence that some essential oils possess anti-inflammatory activity. For example, chamomile essential oil has been used for centuries as an anti-inflammatory and also for alleviating the symptoms associated with eczema, dermatitis and other pronounced irritation [60]. However, there are other examples of essential oils (eucalyptus, rosemary, lavender, millefolia) along with other plants (pine, clove andmyrrh) that have been used as mixed formulations as anti-inflammatory agents [61].

The anti-inflammatory activity of essential oils may be attributed not only to their antioxidant activities but also to their interactions with signalling cascades involving cytokines and regulatory transcription factors, and on the expression of pro-inflammatory genes [62,63].

1.9 Aim of the study

The aims of this project were to :

1. Analyze oil content in the seed of chinotto, in order to assess the profile of fatty acid quantitatively and qualitatively during maturation;
2. Determine antioxidant activities and total phenolic content in MeOH and DMF extracts from the pulps and seeds of chinotto;
3. Compare antioxidant activities and total phenolic content between pulp and seed of Chinotto and analyze their changes during maturation;
4. Analyze the naringin and neohesperidin, as the major flavonoids in fruit of chinotto, contained in pulp during maturation and from different area of cultivation. This study will allow us to know in which stages of the maturation their levels are highest;
5. Isolate the pectin and β-glucan extracted from pulps and seedsof chinotto from different areas of cultivation at various stages of maturation, and characterize them by FT-IR analyses, as a tool to elucidate the structural aspects of the isolated materials. The results of this study will provide the valuable information about the new alternative sources of pectin and β-glucan which is derived from parts of chinotto fruit, such as seeds and pulps;
6. Evaluate the effects of maturation on the composition in volatile compounds of the essential oil from Chinotto peels, and to verify the potential antioxidant and anti-inflammatory properties of these fractions.

CHAPTER 2

RESULTS AND DISCUSSION

2.1 Chinotto seeds

2.1.1 Oil contents

The oil contents of the chinotto seeds at different stage of ripening and area of cultivation are reported as a mean value of three samples (Table 2.1) and are expressed on a dry seed weight flour (5 g). As shown in Table 2.1, the oil increased during maturation both in Calabria and Sicily. At ripe stage the percentage of oil from Sicily seeds (54.8 %) was slightly higher than that contained in Calabria seeds (52.9%).Overripe chinotto from Calabria had the highest content of oil (56.8%), otherwise the lowest content of oil belong to unripe from Calabria (47.7 %).

Table 2.1 Oil contents of the seeds[a]

Sample	Degree of maturation	Area of cultivation	Weight of Oil (g)	w/w (%)
SC02	Unripe	Calabria	23.9 ± 0,5	47.7
SC03	Semiripe	Calabria	24.8 ± 0,7	49.6
SC04	Ripe	Calabria	26.4 ± 1,1	52.9
SC05	Semiripe	Sicily	23.3 ± 0,9	46.5
SC06	Ripe	Sicily	27.4 ± 1,3	54.8
SC07	Overripe	Calabria	28.4 ± 1,5	56.8

[a]Values are mean ± SD of three samples of each species, analysed individually in triplicate.

2.1.2 Fatty acid composition of the oils

The fatty acid compositions of the oils are determined by gas chromatography using methyl tridecanoate as internal standard and are reported in Table 2.2 There are no qualitative differences in the fatty acid profiles of chinotto seed from all areas of cultivation at all ripening stages; the individual fatty acids contents vary according to the climatic conditions and the ripening period. The most represented fatty acids in seed oils of chinotto, both in Calabria and Sicilia seeds, were linoleic, oleic and palmitic acids. The highest amount of linoleic and oleic acids belong to SC05 (6.3–8.5 mg/g_{oil} and 3.8-4.5 mg/g_{oil}, respectively). Moreover, SC06 had the highest content of palmitic acid (3.53-3.96 mg/g_{oil}). At ripe stage, myristic, palmitoleic and behenic acid in Sicily seeds were lower than Calabria seeds; however, in Sicily seeds the content of other fatty acids, such as palmitic, stearic, oleic, linoleic, linolenic, and arachidic acids, was

higher than those in Calabria seeds. At semiripe stage, most ofthe oils from Calabria chinotto seeds contained fatty acids in greater quantities than that from Sicily seeds, except palmitoleic, linoleic and oleic acid. Behenic acid was the least fatty acid in all seed oils of chinotto.

The ripening of chinotto fruit caused the changes in the amount some fatty acids. Palmitic, palmitoleic and behenic acids increased during maturation in Calabria seed oils.Stearic, oleic, linoleic, and linolenic acids decreased during maturation in Calabria seed oils.There were no quantity alteration during maturation in myristic and arachidic acids in Calabria seed oils; their amounts were stable at 0.12 and 0.17 mg/g_{oil}, respectively. In Sicily seeds, the amounts of all fatty acids increased during ripening, except palmitoleic, oleic and linoleic acid whose amount decreased.

Table 2.2 Fatty acid composition of chinotto seeds(mg/g_{oil})[a]

	Fatty Acids	**Sample 1**	**Sample 2**	**Sample 3**
SC02	Myristic Acid (C14)	0.12 ± 0.01	0.11 ± 0.002	0.12 ± 0.03
	Palmitic Acid (C16)	2.68 ± 0.7	2.48 ± 0.8	2.38 ± 0.2
	Stearic Acid (C18:0)	0.39 ± 0.03	0.45 ± 0.10	0.25 ± 0.14
	Oleic Acid (C18:1)	3.55 ± 0.2	3.63 ± 0.5	3.16 ± 0.04
	Linoleic Acid (C18:2)	3.75 ± 0.01	4.43 ± 0.8	3.13 ± 0.18
	Linolenic Acid (C18:3)	1.06 ± 0.01	1.13 ± 0.2	0.83 ± 0.08
	Palmitoleic Acid (C16:1)	0.14± 0.03	0.14 ± 0.6	0.16 ± 0.14
	Arachidic Acid (C20)	0.16 ± 0.06	0.18 ± 0.06	0.17 ± 0.01
	Behenic Acid (C22)	0.02 ±0.005	0.01 ± 0.003	0.01 ±0.002
SC03	Myristic Acid (C14)	0.12 ± 0.003	0.12 ± 0.006	0.12 ± 0.004
	Palmitic Acid (C16)	2.96 ± 0.05	2.03 ± 0.19	2.32 ± 0.10
	Stearic Acid (C18:0)	0.43 ± 0.01	0.42 ± 0.02	0.35 ± 0.01
	Oleic Acid (C18:1)	2.9 ± 0.04	2.97 ± 0.10	2.35 ± 0.08
	Linoleic Acid (C18:2)	4.08 ± 0.06	3.47 ± 0.18	3.28 ± 0.1
	Linolenic Acid (C18:3)	0.94 ± 0,01	0.84 ± 0,01	0.75 ± 0.02

	Palmitoleic Acid (C16:1)	0.05 ± 0.001	0.07 ± 0.002	0.06 ± 0.009
	Arachidic Acid (C20)	0.18 ± 0.001	0.16 ± 0.004	0.18 ± 0.001
	Behenic Acid (C22)	0.03 ± 0.003	0.02 ± 0.003	0.03 ± 0.002
SC04	Myristic Acid (C14)	0.12 ± 0.003	0.12 ± 0.001	0.12 ± 0.005
	Palmitic Acid (C16)	2.7 ± 0.2	3.3 ± 0.02	1.7 ± 0.16
	Stearic Acid (C18:0)	0.04 ± 0.23	0.07 ± 0.009	0.05 ± 0.12
	Oleic Acid (C18:1)	2.8 ± 0.03	3.5 ± 0.09	2.4 ± 0.01
	Linoleic Acid (C18:2)	3.3 ± 0.09	4.02 ± 0.02	3.51 ± 0.15
	Linolenic Acid (C18:3)	0.11 ± 0.07	0.13 ± 0.004	0.15 ± 0.05
	Palmitoleic Acid (C16:1)	0.07 ± 0.007	0.07 ± 0.002	0.06 ± 0.002
	Arachidic Acid (C20)	0.17 ± 0.006	0.17 ± 0.001	0.17 ± 0.001
	Behenic Acid (C22)	0.03 ± 0.005	0.03 ± 0.0001	0.03 ± 0.001
SC05	Myristic Acid (C14)	0.10 ± 0.003	0.11 ± 0.14	0.10 ± 0.02
	Palmitic Acid (C16)	2.4 ± 0.05	2.4 ± 0.11	2.6 ± 0.08
	Stearic Acid (C18:0)	0.05 ± 0.01	0.03 ± 0.02	0.08 ± 0.02
	Oleic Acid (C18:1)	3.8 ± 0.01	4.2 ± 0.17	4.5± 0.02
	Linoleic Acid (C18:2)	6.3 ± 0.03	7.3 ± 0.03	8.5 ± 0.10
	Linolenic Acid (C18:3)	0.13 ± 0.01	0.18 ± 0.1	0.18 ± 0.02
	Palmitoleic Acid (C16:1)	0.15 ± 0.02	0.16 ±0.001	0.19 ± 0.06
	Arachidic Acid (C20)	0.16 ± 0.04	0.16 ± 0.03	0.16 ± 0.05
	Behenic Acid (C22)	0.01 ± 0.001	0.01 ± 0.002	0.01± 0.001
SC06	Myristic Acid (C14)	0.11 ± 0.03	0.11 ± 0.03	0.11 ± 0.03
	Palmitic Acid (C16)	3.53 ± 0.08	3.96 ± 0.38	3.67 ± 0.1
	Stearic Acid (C18:0)	0.45 ± 0.02	0.67 ± 0.07	0.63 ± 0.02
	Oleic Acid (C18:1)	3.24 ± 0.06	3.41 ± 0.31	3.19 ± 0.007
	Linoleic Acid (C18:2)	5.21 ±0.7	5.28 ± 0.5	4.97 ± 0.1
	Linolenic Acid (C18:3)	0.75 ± 0.03	1.13 ± 0.12	1.07 ± 0.03

	Palmitoleic Acid (C16:1)	0.05 ± 0.001	0.03 ± 0.005	0.04± 0.001
	Arachidic Acid (C20)	0.19 ± 0.01	0.20 ± 0.06	0.20 ± 0.02
	Behenic Acid (C22)	0.02 ± 0.009	0.02 ± 0.001	0.02 ± 0.001
SC07	Myristic Acid (C14)	0.12 ± 0.005	0.12 ± 0.003	0.12 ± 0.001
	Palmitic Acid (C16)	2.65 ± 0.03	2.88 ± 0.44	2.76 ± 0.55
	Stearic Acid (C18:0)	0.22 ± 0.06	0.20 ± 0.03	0.21 ± 0.04
	Oleic Acid (C18:1)	3.2 ± 0.005	2.5 ± 0.006	2.9 ± 0.03
	Linoleic Acid (C18:2)	3.88 ± 0.08	3.74 ± 1.01	3.18 ± 0.9
	Linolenic Acid (C18:3)	0.84 ± 0.09	0.91 ± 0.12	0.69 ± 0.05
	Palmitoleic Acid (C16:1)	1.80 ± 0.02	1.96 ± 0.32	1.54 ± 0.2
	Arachidic Acid (C20)	0.17 ± 0.05	0.16 ± 0.005	0.17 ± 0.07
	Behenic Acid (C22)	0.03 ± 0.002	0.03 ± 0.002	0.03 ± 0.005

[a]Values are mean ± SD of three samples of each species, analysed individually intriplicate. Each value is expressed as mg/ g of extracted oil

2.1.3 Biophenol extraction

Biophenol compounds were extracted using two different solvents: MeOH and DMF. The extract yields were reported in Table 2.3 and are expressed on a dry defatted seed weight flour (1 g).

Table 2.3 Biophenol extracts from seeds

Sample	**Degree of maturation**	**Area of cultivation**	**MeOH** (mg/g)	**DMF** (mg/g)
SC02	Unripe	Calabria	70	11
SC03	Semiripe	Calabria	200	80
SC04	Ripe	Calabria	210	90
SC05	Semiripe	Sicily	210	120
SC06	Ripe	Sicily	210	96
SC07	Overripe	Calabria	190	14

The amounts of MeOH extracts were higner than those of DMF extracts at all stages of maturation. MeOH extracts increased during maturation in Calabria seeds, but they did not change in Sicily seeds. Ripe Calabria (SC04) seeds provide the highest amount in MeOH extract. The largest amounts of biophenols from methanol extract were found in ripe seeds from Calabria (SC04), and semi ripe (SC05) and ripe seeds from Sicily (SC06) (210 mg/g defatted seed powder).

In DMF extracts, the yield increased in Calabria seeds during maturation but it gradually decreased when the seeds reached overripe stage. In Sicily seeds, DMF extracts decreased during maturation; semiripe Sicily (SC05) seeds seeds provide the highest amount in DMF extract.

2.1.4 Antioxidant activity

The DMF and methanolic seed extracts were examined and compared for their free radical scavenging activities using DPPH and ABTS assays, whose combination is particularly useful for the screening of plant extract antioxidant capacity [64].

2.1.4.1 DPPHassay

The free-radical scavenging activity of the extracts was evaluatedusing the DPPH method. 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical, and when it reacts with a radicalscavenger, its maximum absorbance at 517 nm fades rapidly. The antioxidant effect is proportional to the disappearance of DPPH in test samples. All the extracts showed a significant antioxidant ability (Table 2.4). The results highlighted that scavenging capacity of the MeOH and DMF extracts on DPPH radicals increased with the concentration. However, the percentage of radical scavenging activity in methanolic extracts were higher than DMF extracts at all concentrations, except for SC02 and SC03 at lower concentration (21.6% and 11.6 % respectively at 0.03 μg/mL), and SC04 at 0.17 and 0.08 μg/mL (69.6 and 47.7%). Both methanolic and DMF extracts of overripe chinotto seeds from Calabria (SC07), showed the highest value of antioxidant ability at lower concentration (35.6% and 14.8%, respectively). It is worth noting that methanolic extract of semi-ripe chinotto (SC05) had the highest percentage of radical scavenging activityat higher concentration (93.1% at 0.33 μg/mL),.

Table 2.4DPPH scavenging ability of the *chinotto* seedextracts

DPPH Scavenging Activity (%)		**SC02**	**SC03**	**SC04**	**SC05**	**SC06**	**SC07**
MeOH Extract	*0.33 μg/mL*	81.6 ± 0.9	80.5 ± 2	90.4 ± 3.3	93.1 ± 2	86.1 ± 2.8	92.1 ± 1.4
	0.17 μg/mL	65.9 ± 5.6	65.1 ± 6.2	66.7 ± 5.3	70.2 ± 7.5	77.2 ± 3.2	81.4 ± 0.9
	0.08 μg/mL	38.3 ± 8.4	38.7 ± 4.4	40.1 ± 1.3	51.4 ± 3.9	41.6 ± 0.3	44.9 ±1.5
	0.03 μg/mL	9.7 ± 3.3	5.6 ± 3.7	25.9 ± 8.7	32.8 ± 4	21.7 ± 4.9	35.6 ± 6.3
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	58.8 ± 0.6	59.2 ± 1.3	65.9 ± 2.1	66.3 ± 1.4	62.6 ± 1.8	65.9 ± 1.1
DMF Extract	*0.33 μg/mL*	78.2 ± 0.1	80.1 ± 2.3	79.3 ± 1.8	70.5 ± 0.3	87.1 ± 6.6	72.3 ± 2.9
	0.17 μg/mL	72.7 ± 1.4	54.0 ± 5.7	69.6 ± 1.7	45.5 ± 1.3	50.7 ± 2.8	45.8 ± 0.8
	0.08 μg/mL	34.7 ± 2.5	24.2 ± 1.5	47.7 ± 1.7	31.8 ± 0.6	35.1 ± 2.4	24.9 ± 1.7
	0.03 μg/mL	21.6 ± 1.7	11.6 ± 4.9	24.8 ± 4.1	2.5 ± 2	8.8 ± 5.1	14.8 ± 1.2
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	55.3 ± 0.1	58.9 ± 1.4	57.6 ± 0.7	51.3 ± 0.2	63.6 ± 3.9	52.6 ± 1.9

The antioxidant activity was also reported in μmol of Trolox equivalents per gram of extract (μmol $TE/g_{extract}$). As shown in Figure 2.1, at the higher concentrationused (0.33 μg/mL), methanolic extract of semi-ripe chinotto (SC05) exhibited a free radical scavenging of 66.28 μmol $TE/g_{extract.}$

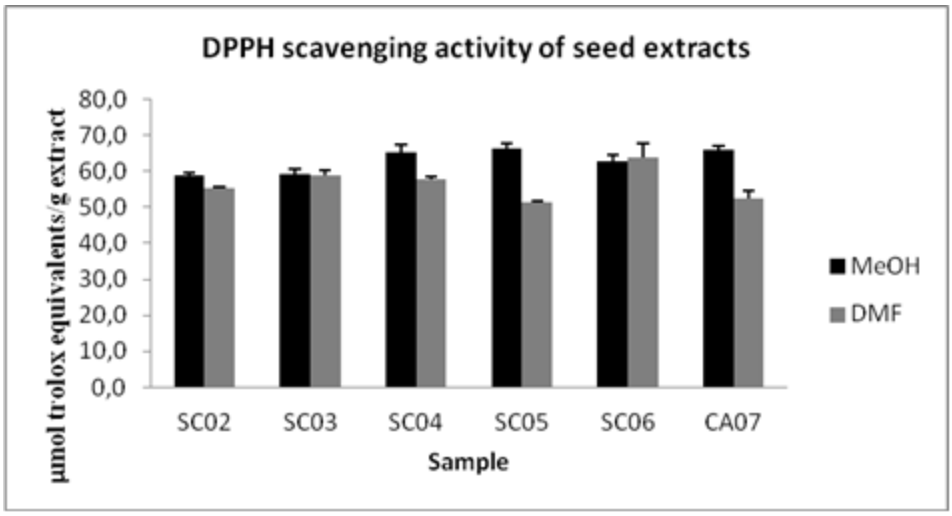

Fig. 2.1 DPPH• radical scavenging activity of chinotto seed extracts. The results are means (±SD) of three separate experiments.The antioxidant activity was reported by µmoles of Trolox equivalents per gram ofdry extract (µmol TE/g extract).

2.1.4.2 ABTS assay

ABTS assay was also applied to measure the antioxidant activity in seed extracts. Data from this experiment were expressed as percentage of antioxidant activity in seed extracts, and were presented as means ± standard deviation (Table 2.5). Antioxidant capacity detected by ABTS assay was lower compared to that by DPPH assay; that extracts from seed had antioxidant ability inferior to 80%. Nevertheless, the results showed that antioxidant capacity by ABTS assay wasstrongly positively correlated to that by DPPH assay. Radical scavenging capacity of methanolic and DMF extracts of chinotto seedson ABTS radical cation increased with the concentration. At the highest concentration (0.33µg/mL), the percentage of radical scavenging activity of DMF extracts from Calabria seeds were higher than those of MeOH extracts. However, the MeOH extracts from Sicily seeds (SC05 and SC06) had a higher values than their DMF extracts at highest concentration (55.9% and 59.1% respectively at 0.33µg/mL). Both methanolic and DMF extracts of overripe chinotto seeds from Calabria (SC07), showed the highest value of antioxidant ability at 0.33µg/mL (67.8% and 79.8%, respectively). At lowest concentration (0.03 µg/mL), DMF extract of unripe seeds (SC02) had the highest percentage of radical scavenging activity (16.6%).

Table 2.5ABTS scavenging ability of the *chinotto* seedextracts

ABTS Scavenging Activity (%)		**SC02**	**SC03**	**SC04**	**SC05**	**SC06**	**SC07**
MeOH Extract	*0.33 μg/mL*	53.4±1.5	62.8±1.5	67.5±1.1	55.9±0.1	59.1±0.5	67.8±1.6
	0.17 μg/mL	30.6±3.4	32.2 ±0.1	36.8 ±1.9	30.1 ±0.4	35.0 ±1.6	36.9 ±0.1
	0.08 μg/mL	14.8 ±0.6	17.8 ±0.2	20.1 ±0.8	18.2 ±2.8	21.6 ±2.1	19.9 ±2.0
	0.03 μg/mL	7.58±0.4	5.83±3.1	10.9 ±1.5	11.2 ±2.1	7.8 ±0.9	5.0 ±1.0
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	29.4 ±1.0	35.4 ±1.0	38.5 ±0.7	31 ± 0.1	33.1 ±0.3	38.8 ± 1.0
DMF Extract	*0.33 μg/mL*	68.6 ±0.4	70.7 ±1.2	71.4 ±0.2	48.7 ±1.3	57.7 ±1.7	79.8 ±6.6
	0.17 μg/mL	38.7 ±2.1	41.4 ±2.4	31.0 ±2.9	24.7 ±1.5	37.8 ±0.8	56.3 ±2.7
	0.08 μg/mL	24.6 ±2.2	20.7 ±1.6	19.8 ±1.0	13 ±0.3	25.2 ±2.4	34.0 ±3.6
	0.03 μg/mL	16.6 ±0.2	14.1 ±0.7	5.8 ±1.3	6.3 ±0.7	8.0 ±2.4	14.5 ±2.3
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	39.1 ±0.2	40.5±0.8	41.0±0.1	26.4 ±0.8	32.3 ±1.1	46.3 ±4.2

The antioxidant capacities were also expressed as micromol Trolox equivalents per gram of extract (TEAC). As shown in Figure 2.2, at the higher concentration used (0.33 μg/mL), methanolic and DMF extracts of overripe chinotto (SC07) exhibited a free radical scavenging of 38.8 and 46.3 μmol $TE/g_{extract}$, respectively.

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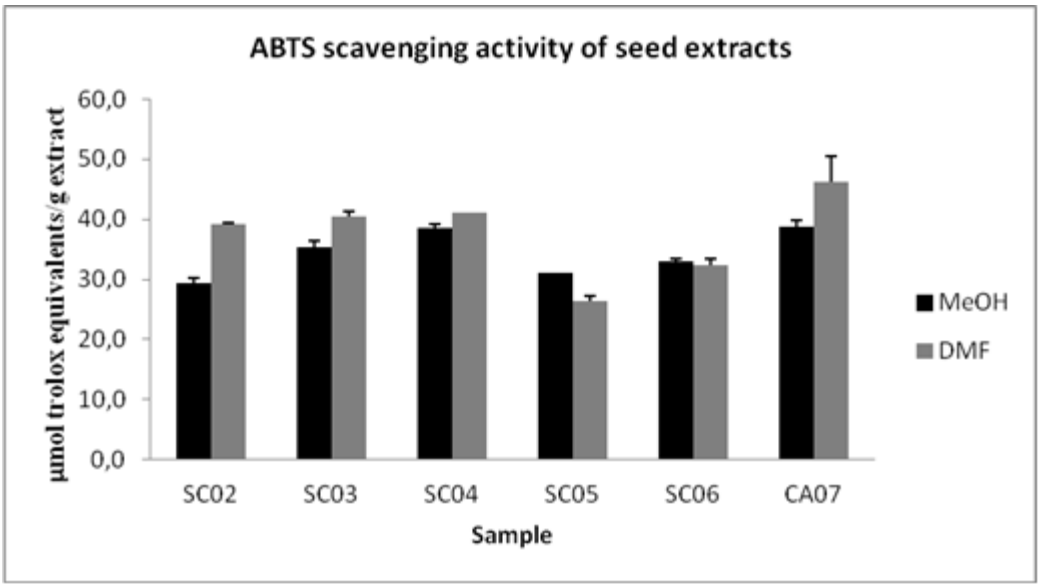


Fig.2.2 ABTS scavenging activity of chinotto seed extracts. The results are means (±SD) of three separate experiments.The antioxidant activity was reported by µmoles of Trolox equivalents per gram ofdry extract (µmol TE/g extract).

2.1.5 Total phenolic content (TPC) of seed extracts

The total phenolic content of each fraction was determined using the Folin-Ciocalteu method with some modification [65] and the results obtained were expressed as mg of gallic acid equivalents per gram of extract (Table 2.6).

Table 2.6 Total Phenolic Content of seed extracts

Sample	MeOH extract (mg GAE/$g_{extract}$)	DMF extract (mg GAE/$g_{extract}$)
SC02	13.5 ± 0.9	1.5 ± 0.1
SC03	44.4 ± 1.1	52.4 ± 0.9
SC04	44.4 ± 0.7	32.3 ± 1.6
SC05	72.5 ± 2.6	80.6 ± 1.4
SC06	24.3 ± 0.7	2.8 ± 0.6
SC07	59.1 ± 2.2	12.2 ± 0.9

The changes of total phenolic content (TPC) of MeOH extracts showed different trends during maturation depending on the regions of origin; it was reduced during ripening for the extracts from Sicily seeds with highest content reached at semi-ripe stage SC05 (72.5mg GAE/$g_{extract}$)

while it increased during ripening for the extracts from Calabria seeds to reach 59.1 mg GAE/g at mature stage (SC07). The total amount of phenolic compounds detected in the seed extracts were directly related to the values of radical scavenging activity.As the methanolic extract, DMF extracts of SC05 also had a highest value (80.6 mg GAE/$g_{extract}$). These results indicated that total phenolic content in seed residue extracts was strongly correlated with antioxidant activity, suggesting that phenolic compounds contribute to their antioxidant capacities.

2.1.6 Pectins

The pectins were extracted under 3 different pH conditions, in order to monitor the effect of extraction pH on the pectin yield and their degree of methoxylation. The results showed the differences in yields and degree of methoxylation during maturation under different pH values. The percentage yields of pectin from chinotto seeds increased during ripening in all fractions (Table 2.7). Ripe and overripe seeds from Calabria chinotto provided more pectins A (4.59% and 5.96 %, respectively) than ripe seeds from Sicily (3.83%).

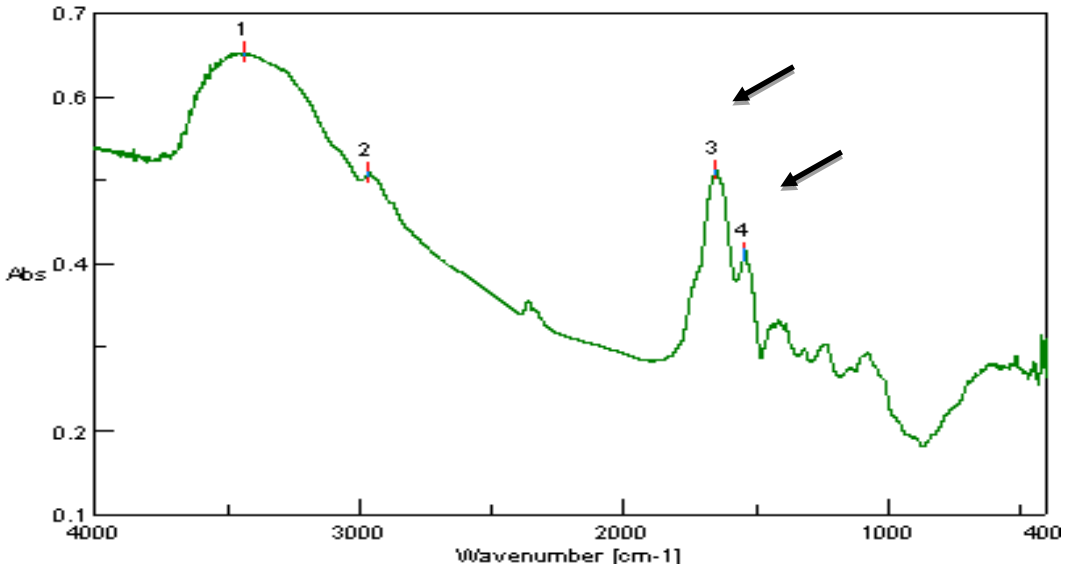
Table 2.7 Pectin yield obtained from chinotto seeds

	Yield (%)					
Fractions	SC02	SC03	SC04	SC05	SC06	SC07
A	2.6	3.3	4.6	3.4	3.8	5.7
A^-	11.4	11.8	12.4	21.1	40.6	12.5
A^+	15.8	17.1	18.7	34.8	57.1	21.3

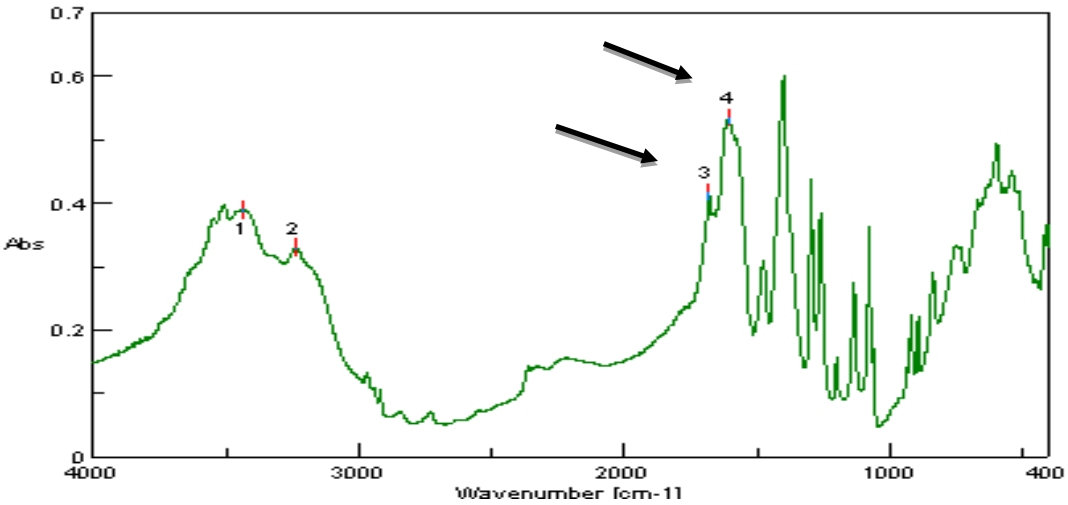
2.1.6.1 Characterization of pectins

The degree of methoxylation (DM) of the pectin is the important factors in determining the texture of the gel. Fruits with high DM can be used for the production of jams and marmalades. Generally, the ingredients required for the formation of the gel may be present in the fruit itself: 65% of sugar, acidity around 3.0 and higher DM (>50%). Different pH values of extraction effected the change of degree of methoxylation.Conditions of extreme acidity (pH 1.0) caused a hydrolysis of the methyl esters and a de-polymerization of pectin chain (Fraction A^-, [66]). The same situation also occurred in extreme alkaline (pH 12.0) conditions, which caused saponification of methyl esters groups and the production of different sized pectin chains by β-

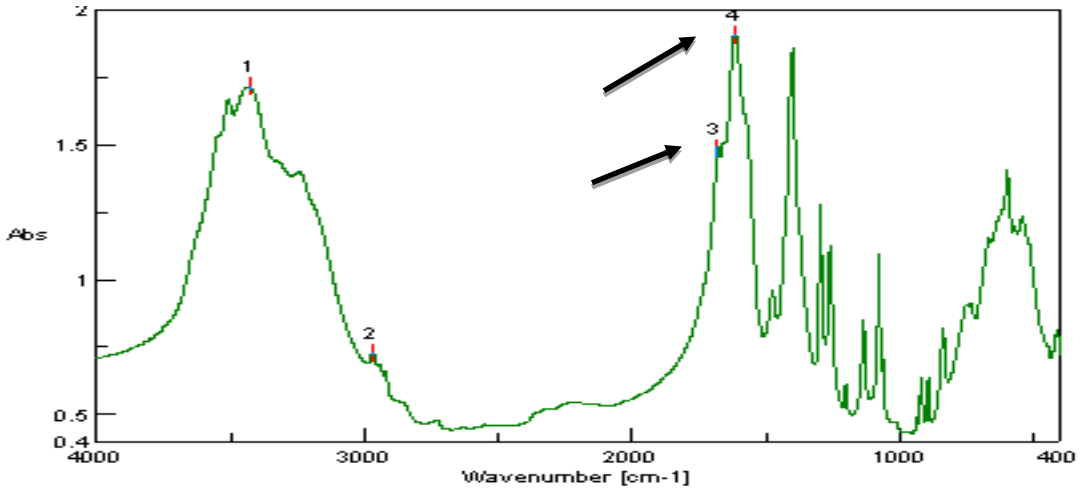
elimination (Fraction A^+, [67]). Both treatments can cause a decrease in the DM of pectins.The variation in degree of methoxylation was related with the pH. Fractions A, A^- e A^+ of chinotto seeds were characterized by FT-IR spectroscopy (Figure 2.3 (a), (b), (c), respectively): the band at 1631 cm^{-1} (peak number 4 in fig 2.3) corresponds to the symmetrical stretching vibration of free carboxyl group, while the peak at 1741 cm^{-1} (peak number 3 in fig. 2.3) was assigned to methyl ester group. Each pectin fraction obtained at different pH showed the same qualitative profile at all stages of ripening. After the analysis of each spectrum through identification of the two peaks, The DM was obtained using the equation [Abs 1741 cm^{-1}/ (Abs 1631 cm^{-1} + Abs 1741 cm^{-1})] [68].



a. Infrared spectrum of fraction **A**



b. Infrared spectrum of fraction $\mathbf{A^-}$



c. Infrared spectrum of fraction $\mathbf{A^+}$

Fig. 2.3 Infrared spectrum of pectins extracted from chinotto seeds at different pH

The spectra presented in Fig. 2.3 confirmed that the variation in the degree of methoxylation was associated with the pH (Table 2.8). DM in pectin from seeds increased during the maturation (table 2.8), in the case of fraction A, both in seed from Calabria and Sicily. The highest DM was obtained for ripe seeds from Sicily (SC06, 72.7%). DM decreased during maturation in fractions A^- (Calabria and Sicily) and A^+ (Sicily), but increased in fraction A^+ Calabria seeds.

Table 2.8 Degree of methoxylation of pectins in chinotto seeds

	Degree of Methoxylation (%)					
Fraction	SC02	SC03	SC04	SC05	SC06	SC07
A	40.3 ± 1.2	44.7 ± 2.2	50.3 ± 1.2	58.5 ± 3.0	72.7 ± 1.9	55.3±1.3
A^-	37.3 ± 1.8	33.7 ± 3.1	47.7 ± 2.3	45.2 ±1.3	42.2 ±2.3	31.7 ± 3.7
A^+	32.7 ± 1.2	30.5 ± 1.1	42.9 ±1.7	45.4 ± 1.7	42.2 ± 3.6	45.8 ± 2.4

2.1.7 β-glucans

The content of β-glucan in Chinotto seeds were reported in Table 2.9. The amount of β-glucans decreased during maturation; seeds of unripe Calabria chinotto (SC02) contained the highest amounts of β-glucans (6.80%), this value higher than β-glucan content in barley flour with only 4.75 % [69]. The smallest content belong to SC07 (1.39%). Seeds from Calabria had a lower amount of β-glucans than those from Sicilia at the same degree of maturation.

Table 2.9 The amount of β-glucans contained in chinotto seeds

Sample	**Degree of Maturation**	**β-glucans** (%)
SC02	Unripe Calabria	6.8
SC03	Semiripe Calabria	1.7
SC04	Ripe Calabria	1.5
SC05	Semiripe Sicily	2.1
SC06	Ripe Sicily	1.9
SC07	Overripe Calabria	1.4

2.1.7.1 Characterization of β-glucans by Infrared Transmission Spectroscopy

Infrared spectroscopy allows the measurement of molecular vibrations of covalent bonds. The IR region 4000-400 cm^{-1} provides information on the fundamental vibrations. The infrared spectra

from commercial and extracted β-glucans were shown in Figures 2.4 and 2.5, respectively. The FT-IR spectra of all seeds have the same profile; there were no changes during maturation and from different area of cultivation.

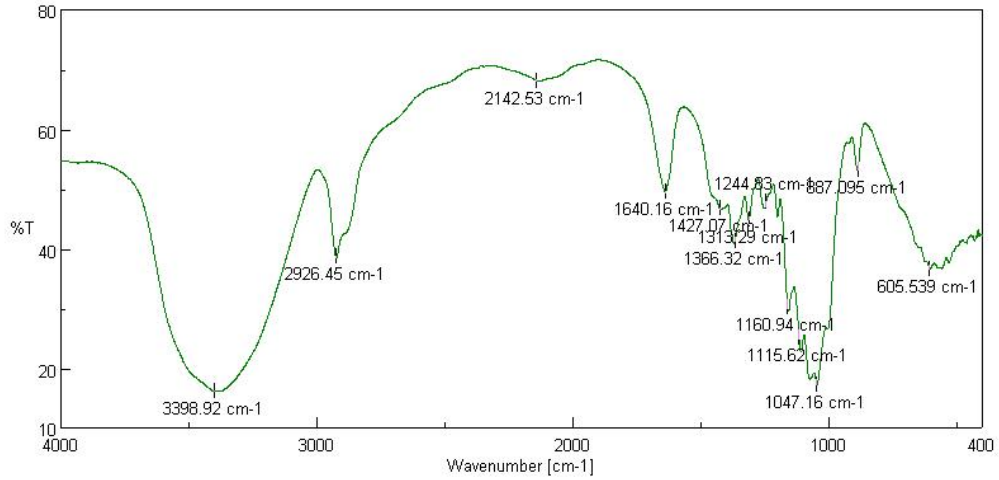


Fig. 2.4 Infrared spectrum of commercial β-glucans

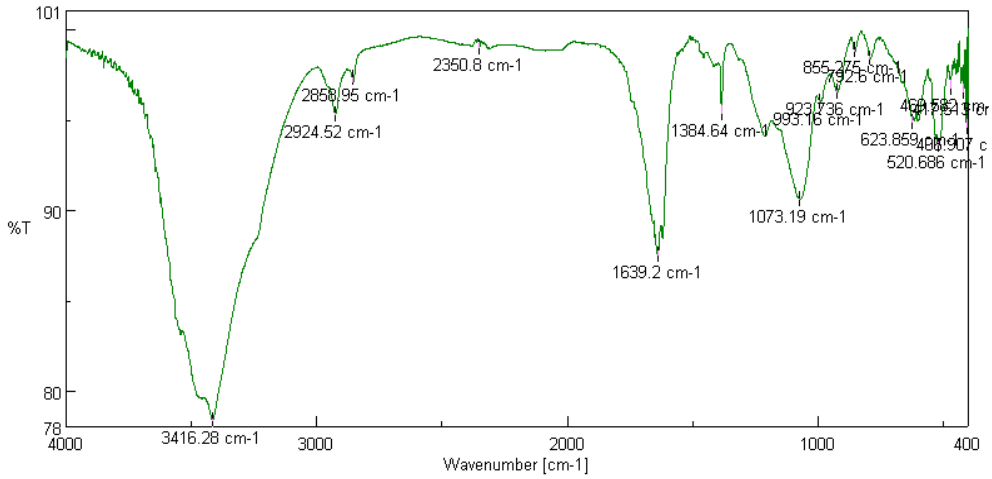


Fig. 2.5 Infrared spectrum of extracted β-glucanfrom chinotto seed

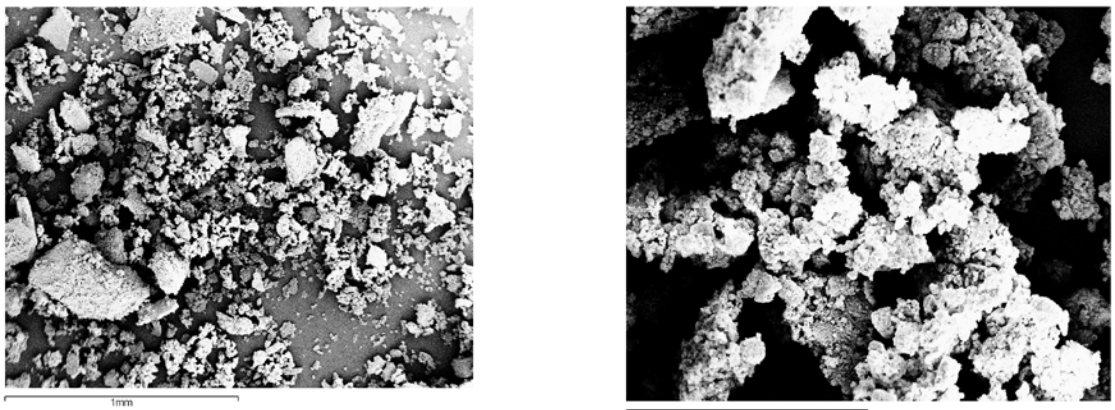
In the region of 4000–3000 cm^{-1}, the extracted and commercial β-glucans spectra showed a wide band with maximum absorption (minimum transmittance) at 3416 cm^{-1} and 3398 cm^{-1}, respectively. This can be attributed to normal vibration modes of asymmetric and symmetric stretching of OH groups because polysaccharides contain a significant number of OH groups, which exhibit an absorption band above 3000 cm^{-1}. The absorption peaks occurring at 2924 cm^{-1}

(extracted) and 2926 cm^{-1} (commercial) in the region of 3000–2840 cm^{-1}could be attributed to the relative values of the vibrational modes of asymmetric and symmetric stretches of CH groups [70]. The strong absorption at 1639 cm^{-1} for extracted β-glucans and 1640 cm^{-1} for commercial β-glucans were due to the stretching of CN groups and NH groups of the proteins indicating the presence of amide linkages and the presence of protein in the sample. The region 1285–242 cm^{-1}, which showed peaks with maximum absorption at 1073 cm^{-1} (extracted) and 1047 cm^{-1}(commercial), corresponds to COC and CO bonds of a ring of D-glucose [71], which are network vibrations in which all of the atoms of the macromolecular chain vibrate in phase and normal modes resulting from coupling of the CC and CO stretches. Therefore, the peaks at 1073 cm^{-1} for the extracted sample and at 1047 cm^{-1} for the commercial one indicate the presence of glycosidic bonds and cyclic structures of monosaccharides.

Carbohydrates can be recognized by peaks at wave numbers of 1040 cm^{-1} (CO bond of the alcohol group), 2940 cm^{-1} (CH stretch) and 3400 cm^{-1} (OH stretch) [70]. It is also important to note that the spectra showed absorption peaks at 993 cm^{-1} for the extracted sample and 887 cm^{-1} for the commercial sample, which is indicative of a β-glycosidic anomeric bonds.

2.1.7.2 Scanning electron microscopy of β-glucan from the seeds of chinotto

The morphology of extracted β-glucans at all stages of maturation was similar. A panoramic view of the samples (Fig.2.6a) revealed that extracted β-glucans had an irregular and small particle size distribution. A few clusters of rounded structure and small loose particles with geometric shapes.A higher magnification of a cluster of the extracted sample (Fig. 2.6b) showed some porous appearances and rough surfaces.



A. 50x **B. 500x**

Fig. 2.6 Scanning electron micrographs of extracted β-glucans from seeds of chinotto.

2.2 Pulps of Chinotto

2.2.1 Biophenol extraction

Biophenol compounds in pulp were also extracted using two different solvents: MeOH and DMF. The extract yields were reported in Table 2.10 and are expressed on a dry pulp weight flour (1 g).

Table 2.10 Biophenol extracts from pulps

Sample	Degree of maturation	Area of cultivation	MeOH (mg/g)	DMF (mg/g)
PC02	Unripe	Calabria	420.1	417.7
PC03	Semiripe	Calabria	422.7	535.5
PC04	Ripe	Calabria	493.2	530.3
PC05	Semiripe	Sicily	467.5	481.64
PC06	Ripe	Sicily	488.7	575.6
PC07	Overripe	Calabria	528.9	550.1

The amount of MeOH extracts from pulps increased during maturation both in Calabria and Sicily. In DMF extracts, the amount of extracts was increased during maturation and it occurred in Sicily and Calabria pulps. The amounts ofDMF extracts were higher than those of MeOH extracts in all stages of maturation except unripe Calabria (PC02). The lowest amount in MeOH extracts was provided from the pulp of unripe Calabria chinotto (420.1 mg/g) and the lowest amount in DMF extracts was unripe Calabria (PC02) (417.7 mg/g).

2.2.2 Antioxidant activity

The DMF and methanolic extracts from pulp were examined and compared for their free radical scavenging activities using DPPH and ABTS assays.

2.2.2.1 DPPH assay

The methanolic and DMF seed extracts of chinotto pulps were examined and compared for their free radical scavenging activities against DPPH (Table 2.11). All pulpextracts exhibited an antioxidant ability. MeOH extracts had antioxidant activity higher than DMF extract, except

those extracted from semiripe Calabria pulp (PC03) at 0.17μg/mL and 0.08μg/mL, and overripe Calabria pulp at 0.33 μg/mL and 0.03 μg/mL.At the lowest concentration (0.03 μg/mL), unripe Calabria (PC02) still had high activity than the others (15%). DMF extract of the pulpfrom ripe Calabria chinotto (PC07) had the highest radical scavenging ability at the highest concentration (81.7% at 0.33μg/mL).

Table 2.11 DPPH scavenging ability of the *chinotto* pulp extracts

DPPH Scavenging Activity (%)		**PC02**	**PC03**	**PC04**	**PC05**	**PC06**	**PC07**
MeOH Extract	*0.33 μg/mL*	76.2 ± 4.2	76.1± 3.3	79.5 ± 3.9	78.8 ± 0.2	80.9 ± 3.6	78.4 ± 2.9
	0.17 μg/mL	56 ± 3.3	49.3 ± 2.1	63.3±5.2	56.7 ± 5.8	58.9 ± 2.1	53.1 ± 4.9
	0.08 μg/mL	22.9 ± 3.7	25.9 ± 7.2	33.7 ± 4.1	42.3 ± 3.2	37.7 ± 9.3	37.3 ± 1.1
	0.03 μg/mL	15 ± 3	9.2 ± 0.4	14.5 ± 0.6	12.3 ± 0.6	8.4 ± 7.3	9.4 ± 7.2
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	56.03 ± 2.7	56.1 ± 2.1	56.3 ± 2.7	55.8 ± 0.2	59.1 ± 2.3	56.6 ± 1.2
DMF Extract	*0.33 μg/mL*	73.4 ±0.3	74.5 ± 1.5	79 ± 0.8	75.8 ± 1.6	78.3 ± 1	81.7 ± 3.5
	0.17 μg/mL	52 ± 1.9	56.8 ± 5.9	54.9 ± 2.1	55.9 ± 6.4	55.3 ± 3.4	51.9 ± 0.3
	0.08 μg/mL	22.8 ± 5.2	35.1 ± 0.8	31±2	34.5 ± 8.2	27.4 ± 0.8	31± 3.7
	0.03 μg/mL	11.3 ± 2.9	7.3 ± 3.6	4.2 ± 3.3	1.4 ± 0.6	2.3±1.8	12.8 ± 0.8
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	54.2 ± 0.2	55.2 ± 0.9	56.6 ± 1.6	55.1 ± 1.0	56.2 ± 0.7	58.9 ± 2.4

The antioxidant capacities from pulp extracts were also expressed as micromol Trolox equivalents per gram of extract (TEAC). As shown in Figure 2.7 the scavenging capacity of the DMF extracts on DPPH radicals increased with the concentration while the antioxidant capacityof the methanolic extracts increased greatly with the concentration in Sicily chinotto pulps and slightly in Calabria chinotto pulps. The highest antioxidant activity belonged to methanolic extracts of pulp from ripe Sicily Chinotto PC06 (59.6 ± 1.2 μmol TE/g extract).

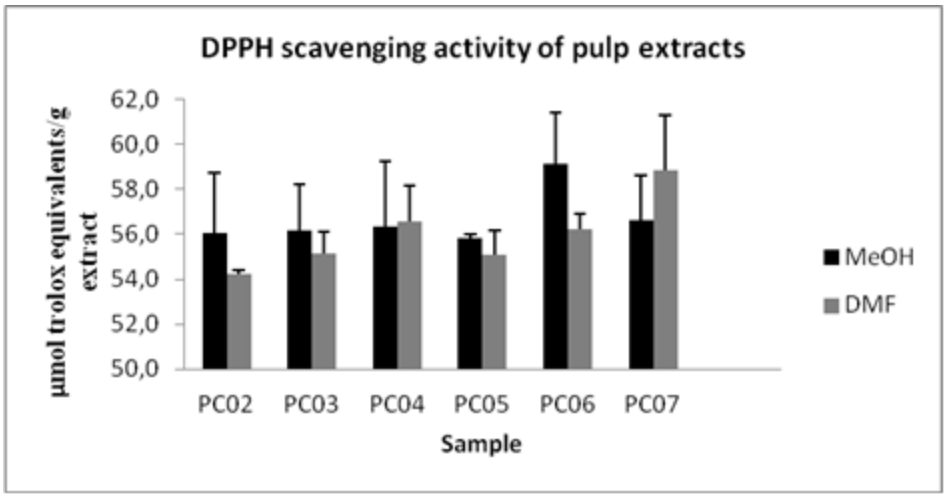


Fig. 2.7 DPPH• radical scavenging activity of chinotto pulp extracts. The results are means (±SD) of three separate experiments.The antioxidant activity was reported by µmol of Trolox equivalents per gram ofdry extract (µmol TE/g extract).

2.2.2.2 ABTS assay

ABTS assay was also applied to measure the antioxidant activity in pulp extracts. Data from this experiment were expressed as percentage of antioxidant activity in seed extracts, and were presented as means ± standard deviation (Table 2.12). This experiment showed that the reduction of ABTS radical cations tend to be less efficient than that DPPH· radical. Table 2.12 showed that ABTS scavenging ability of the chinotto pulp extracts was lower than those measured against DPPH. In this assay, all extracts from pulps exerted antioxidant ability inferior to 67%. The results showed that ABTS scavenging capacity of the methanolic extracts and DMF extracts of chinotto pulps on DPPH radicals increased with the concentration. At the highest concentration (0.33 µg/mL), the percentage of radical scavenging activity ofDMF extracts from Calabria pulps were higher than that of MeOH extracts. However, the MeOH extracts from Sicily pulps had higher values than their DMF extracts at concentration 0.33 µg/mL. At the lowest concentration (0.03 µg/mL), semiripe chinotto pulps (PC03) had the highest percentage of radical scavenging activity both in their DMF and MeOH extracts (22.81%and 21.77%, respectively).

Table 2.12 ABTS scavenging ability of the chinottopulpextracts

ABTS Scavenging Activity (%)		**PC02**	**PC03**	**PC04**	**PC05**	**PC06**	**PC07**
MeOH Extract	*0.33 μg/mL*	48.9±1.3	49.0±4.1	58.5±0.4	54.6 ±1.0	58.7 ±1.4	56.3 ±0.3
	0.17 μg/mL	30.6±3.4	41.4±0.5	37.9±3.6	43 ±0.2	38.7 ±0.5	33.0 ±1.1
	0.08 μg/mL	27 ±0.9	32.5 ±0.5	30.2 ±2.1	28.7 ±0.4	22.7 ±0.1	21.4 ±0.2
	0.03 μg/mL	13.5 ±0.4	21.8 ±1.6	17 ±0.9	20.7 ±0.4	11.5 ±1.3	13.0 ±0.5
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	26.7±0.8	26.8±2.6	32.5±0.2	30.7±0.6	32.6±0.9	31.0±0.2
DMF Extract	*0.33 μg/mL*	53.1 ±1.1	52.9 ±1.6	58.3 ±0.9	49.7 ±2.2	50.7 ±0.1	57.5 ±0.3
	0.17 μg/mL	36.4 ±2.3	34.1 ±0.9	42.7 ±0.4	39.7 ±0.9	30.0 ±1.9	38.7 ±0.8
	0.08 μg/mL	28.9 ±1.6	28.5 ±2.5	18.1±0.9	29.2 ±0.6	21.3 ±0.6	27.2 ±0.2
	0.03 μg/mL	11.7 ±1,6	22.8 ±0.8	11.9 ±2.1	19.7±1.4	13.7 ±0.6	10.7 ±2.5
(μmol $TE/g_{extract}$)	*0.33 μg/mL*	29.3±0.7	29.2±1.0	32.5±0.6	27.9±1.3	27.5±0.1	31.9±1.2

The antioxidant capacities against ABTS were also expressed as micromol Trolox equivalents per gram of extract (TEAC). As shown in Figure 2.8 the scavenging activity in MeOH extracts increased during development of fruit until they reached ripe stage both in Calabria and Sicily pulps. However, the value in Calabria pulps decreased when they turned into overripe. The highest antioxidant capacity of MeOH extracts was provided by ripe Sicily pulps (32.6 μmol $TE/g_{extracts}$) and the lowest value by unripe Calabria pulps (26.7μmol $TE/g_{extracts}$).

The radical scavenging activity in DMF extracts from Calabria chinotto pulps had the same pattern as in MeOH extracts which increased until ripe and started to decrease when overripe. Among DMF extracts, ripe Calabria pulps provided the highest value of antioxidant capacity (37.6μmol $TE/g_{extracts}$), while ripe Sicily pulps the lowest capacity (27.5 μmol $TE/g_{extracts}$).

DMF extracts of Calabria chinotto had higher value of antioxidant ability than MeOH extracts in all stages. Otherwise, in MeOH extracts of Sicily pulps the values of antioxidant ability were higher than those in DMF extracts at all stages.

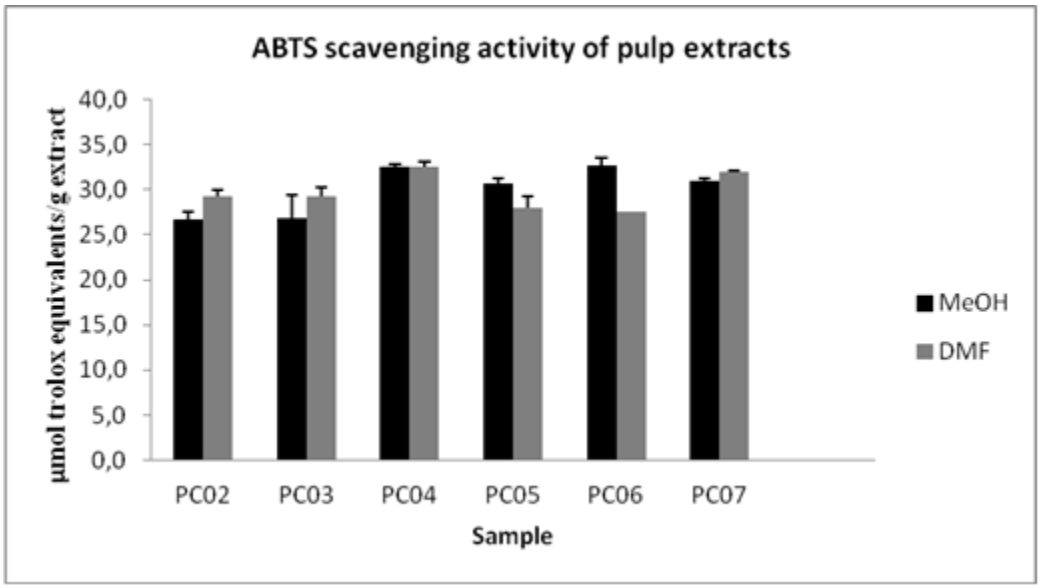


Fig. 2.8ABTS scavenging activity of chinotto pulp extracts. The results are means (±SD) of three separate experiments.The antioxidant activity was reported by µmoles of Trolox equivalents per gram ofdry extract (µmol TE/g extract).

2.2.3Total phenolic content (TPC) of pulp extracts

Total phenolic content of pulp extracts was estimated using the Folin-Ciocalteu method and expressed as mg of gallic acid equivalents per gram of extract (Table 2.13).

Table 2.13 Total Phenolic Content of pulp extracts

Sample	MeOH extract (mg GAE/$g_{extract}$)	DMF extract (mg GAE/$g_{extract}$)
PC02	53.8 ± 1.2	71.2 ± 1
PC03	55.1 ± 2.3	75.2 ± 1.2
PC04	69.9 ± 1.3	77.9 ± 1.3
PC05	47.1 ± 1.4	83.3 ± 2.2
PC06	67.2 ± 1.2	92.7 ± 1.3
PC07	130.2 ± 2.1	104.7 ± 2.4

Overripe Calabria Pulp (PC07) had the highest value of phenolic contents both in MeOH and DMF extracts (130.2 mg GAE/g and 104.7 mg GAE/g, respectively). The lowest content was from MeOH extract of semiripe Sicily and unripe Calabria. Total phenolic content in pulp of chinotto increased during ripening for MeOH and DMF extracts. These results were correlated with the antioxidant tests of the pulp.

2.2.4 Naringin and Neohesperidin contents

In this work were determined the amounts of naringin and neohesperidin contained in the pulp (Table 2.14) since previous study [8,9,10] showed that naringin and neohesperidin are the most abundant flavonoids in chinotto. Chinotto pulp contained more naringin than neohesperidin in all extracts. In MeOH extraction, the value of naringin increased during ripening both in Calabria and Sicily pulps. Nevertheless, when Calabria pulp reached level of overripe, the values decreased. This phenomenon occurred in naringin and neohesperidin from all Calabria pulp extracts, both DMF and MeOH. The concentration of neohesperidin in MeOH extracts from Sicilia decreased during ripening. The highest value of naringin and neohesperidin in MeOH extract was ripe Calabria (PC04) (0.31 mg/g for both naringin and neohesperidin contents). Moreover, the lowest of naringin and neohesperidin was unripe Calabria (PC02) (0.22 and 0.06 mg/g, respectively).

In DMF extracts, the value of naringin and neohesperidin from Sicily pulps increased during maturation; the highest quantity of naringin was contained in DMF extracts of unripe Calabria pulp (PC02) and the lowest in semiripe Sicily (PC05) (0.33 and 0.20 mg/g, respectively). DMF extracts of ripe Calabria pulps (PC04) had the highest amount of neohesperidin (0.23 mg/g) and those of semiripe Sicily pulps had the lowest content (0.13 mg/g).

In Calabria pulps, DMF extracts had a major content of naringin and neohesperidin than MeOH extracts. Otherwise, MeOH extracts from Sicily pulps provided a higher content of naringin and neohesperidin than DMF extracts.

Table 2.14 Naringin and Neohesperidin contents ($mg/g_{extract}$) in pulp of chinotto[a]

	MeOH extracts		DMF extracts	
Sample	Naringin	Neohesperidin	Naringin	Neohesperidin
PC02	0.22±0.01	0.06±0.01	0.33±0.02	0.20±0.02
PC03	0.30±0.01	0.22± 0.08	0.30±0.05	0.20±0.01
PC04	0.31±0.01	0.31±0.02	0.32±0.03	0.23± 0.03
PC05	0.23±0.01	0.21±0.004	0.20±0.01	0.13±0.03
PC06	0.26± 0.04	0.16±0.05	0.24±0.01	0.16±0.05
PC07	0.27 ± 0.02	0.29±0.07	0.29±0.02	0.2 ± 0.05

[a]The results are means (±SD) of three separate analyses.

2.2.5 Determination of pectin in pulps

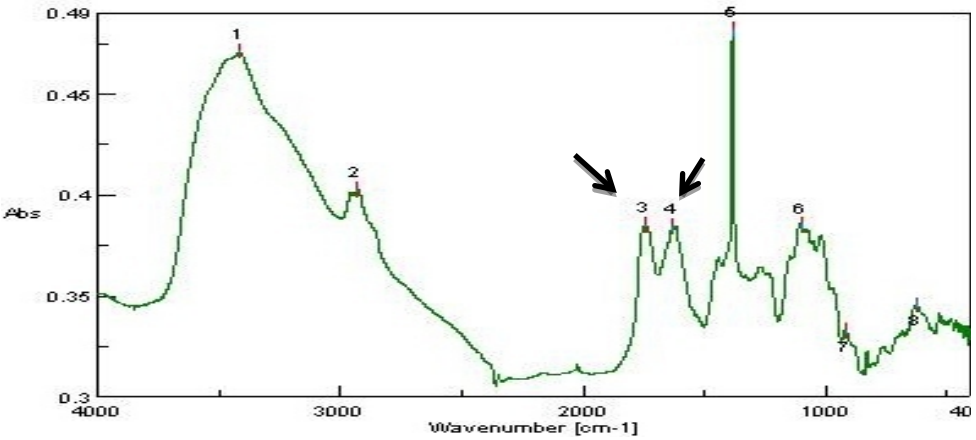
The data reported in literature showed that overripe fruits contain poorer pectin [43]. Force-induced failures of intercellular adhesion are seen in some ripe or overripe fruits, in heated tissues, and in special areas such as abscission zones. In these cases they have usually been changes that resulted in a weakening of the middle lamella and degradation of its pectin component [44]. In agreement with the literature the amounts of pectin decreased during maturation both in chinotto from Calabria and Sicily, and it occurred at all pH conditions (table 2.15). The highest yield of pectins was obtained as a result of the alkaline extraction at pH 12.0 (A^+). At extreme acidity condition (pH 1.0), unripe Calabria and semiripe Sicily pulps had more pectin content (7.7%, 4.4%, respectively) than the mother fraction A (5.3% and 4.0%, respectively).

Table 2.15 Pectin yield obtained from chinotto pulps

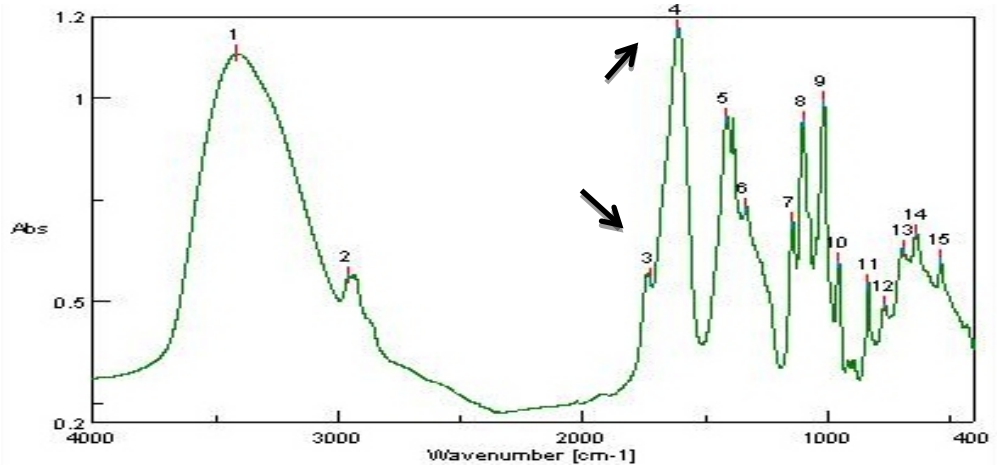
	Yield (%)					
Fractions	SC02	SC03	SC04	SC05	SC06	SC07
A	5.3	4.2	4.1	4.0	3.2	3.1
A^-	7.7	3.6	3.2	4.4	2.6	2.6
A^+	30.3	16.9	12.2	11.2	5.7	6.3

2.2.5.1 Characterization of pectins

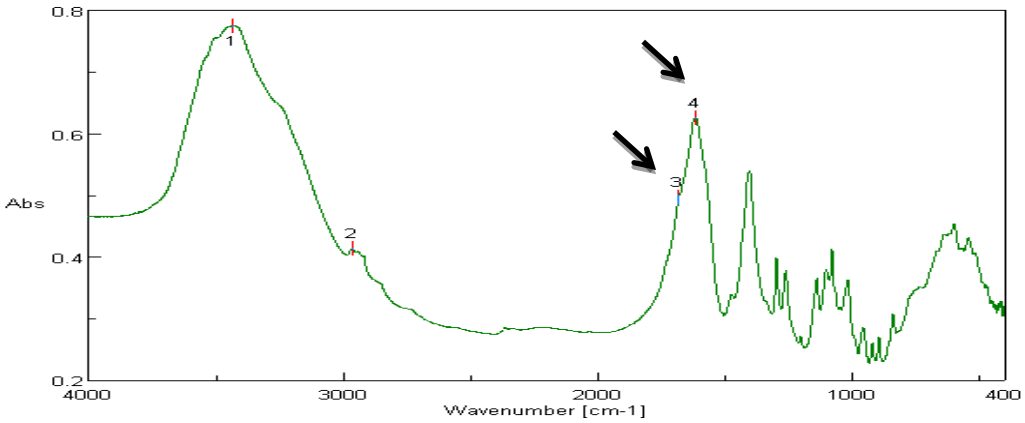
As well as made for the seed pectins, fractions A, A^- e A^+ of chinotto pulps were characterized by FT-IR spectroscopy (Figure 2.9)



a. Infrared spectrum of fraction **A**



b. Infrared spectrum of fraction $\mathbf{A^-}$



c. Infrared spectrum of fraction $\mathbf{A^+}$

Fig. 2.9 Infrared spectrum of pectins extracted from chinotto pulps at different pH.

The values of DM in pectins from pulps were reported in Table 2.16. Each pectin fraction obtained at different pH showed the same qualitative profile at all stage of ripening. In fraction A (mother fraction), the degree of methoxylation of pectins in Calabria chinotto pulps decreased during maturation. However, it slightly increased during maturation in pectins obtained from Sicily chinotto pulps. The highest DM was obtained for ripe pulps from Sicily (PC06, 52.6%). DM increased in fractions A^- and A^+ during maturation, except fraction A^+ from Calabria.

Table 2.16 Degree of methoxylation of pectins in chinotto pulps

	Degree of Methoxylation (%)					
Fraction	PC02	PC03	PC04	PC05	PC06	PC07
A	49.5 ± 0.2	48.7 ± 0.5	48.2 ± 0.4	49.8 ± 1.3	52.6 ± 0.2	48.1 ± 0.4
A^-	43.4 ± 0.9	40.4 ± 1.9	34.5 ± 1.2	34.5 ± 3.0	45.6 ± 1.3	46.8 ± 2.4
A^+	47.0 ± 0.9	43.7 ± 2.2	46.5 ± 1.0	45.6 ± 0.6	46.1 ± 1.5	46.0 ± 0.3

2.2.6 β-glucans

The content of β-glucan in Chinotto pulps were reported in Table 2.17. The amount of β-glucans decreased in Calabria pulps during maturation but increased in Sicily pulps. The highest content of β-glucan was obtained from unripe Calabria pulp (PC02, 0.42%). However, the lowest

percentage of β-glucan was from semiripe chinotto pulps of Sicily (PC05, 0.21%). At the ripe stage, chinotto pulp from Sicily (PC06) had the highest content of β-glucan (0.35 %).

Table 2.17 The amount of β-glucans contained in chinotto pulps

Sample	**Degree of Maturation**	**β-glucans** (%)
PC02	Unripe Calabria	0.42
PC03	Semiripe Calabria	0.37
PC04	Ripe Calabria	0.31
PC05	Semiripe Sicilia	0.21
PC06	Ripe Sicilia	0.35
PC07	Overripe Sicilia	0.28

The infrared spectrum of extracted β-glucans from the pulps was shown in Figure 2.10. The FT-IR spectra of all pulps have the same profile; there were no changes during maturation and from different area of cultivation. The IR spectrum of the pulps showed the characteristic bands of β-glucans such as seed IR spectra: a wide band with maximum absorption at 3406 cm^{-1} attributed to normal vibrational modes of asymmetric and symmetric stretching of OH groups; the peak at 2920 cm^{-1},attributed to asymmetric and symmetric stretches of CH groups; the strong absorption at 1641 cm^{-1} due to the stretching of CN groups and NH groups of the proteins and the peak at 1070 cm^{-1} that corresponds to COC and CO bonds of a ring of D-glucose. The last spectra showed absorption peaks at 916 cm^{-1}, which was indicative of a β-glycosidic anomeric bonds.

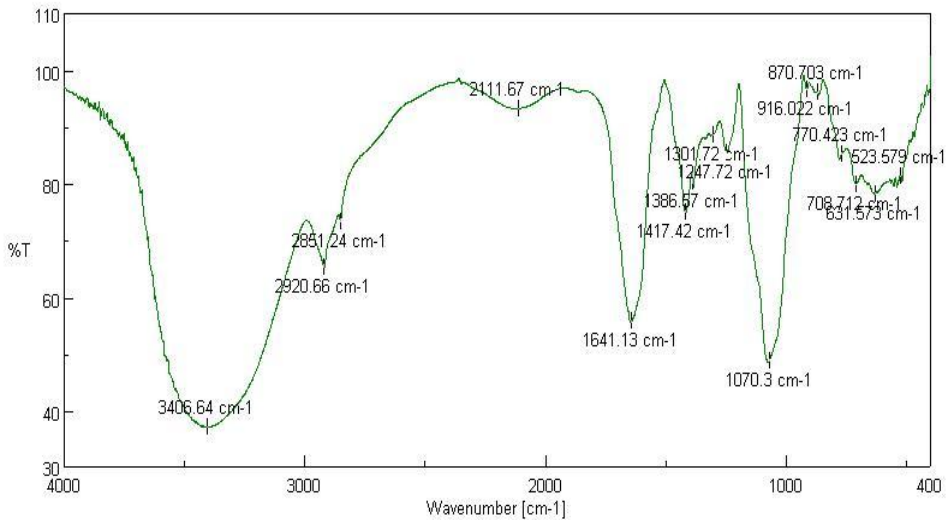
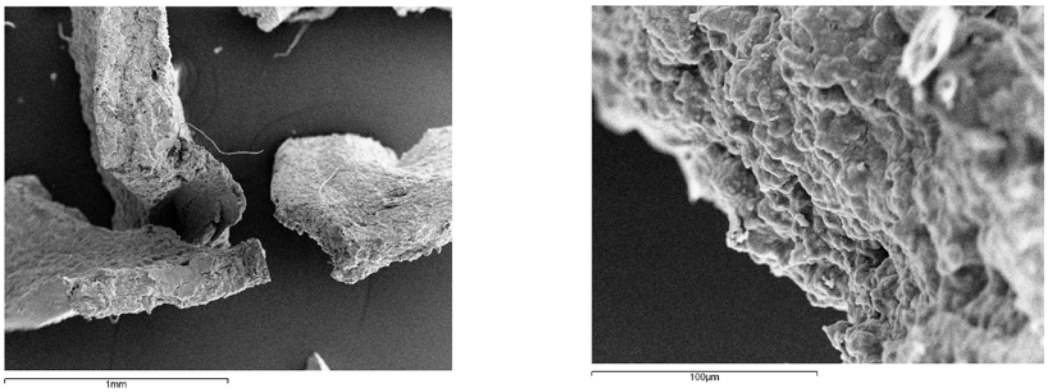


Fig. 2.10 Infrared spectrum of β-glucanextracted from chinotto pulps

2.2.6.1 Scanning electron microscopy of β-glucan from the pulps of chinotto

The morphology of extracted β-glucan from the pulps of chinotto was similar at all stages of maturation. β-glucans from the pulp had geometric shapes with a porous and spongy appearance (Fig.2.11)



A. 50x **B. 500x**

Fig. 2.11 Scanning electron micrographs of extracted β-glucans from pulp of chinotto

2.3 Volatile compounds, antioxidant and anti-inflammatory properties of the essential oil from Chinotto peels

The composition of essential oil from ripe Chinotto has been already reported [72]. However, the effect of maturation on the volatile compounds from Chinotto has not been investigated. Moreover, despite its taxonomically close relation to bergamot (*Citrus bergamia* Risso) [73], whose essential oil properties are well known [74], the potential antioxidant and anti-inflammatory effects of Chinotto essential oil have never been investigated.

The objectives of this work were therefore to evaluate the effects of maturation on the composition in volatile compounds of the essential oil from Chinotto (*Citrus Myrtifolia* Raf.), and to verify the potential antioxidant and anti-inflammatory properties of these fractions.

Fruits were picked-up in the period October - December 2012 and were classified, as unripe (CEO1), semiripe (CEO2) and ripe (CEO3), on the basis of their ripening stage. Essential oils were obtained by cold-pressing the peels and then analyzed by gas-chromatography. The results are reported in Table 2.18 Available data from literature are also included for comparison.

Limonene, linalool and linalyl acetate resulted the main components in all the oils analyzed (CEOs). In particular, limonene content increased with maturation, reaching its highest level in the CEO2 and then slightly declining. Linalool contents increased with maturation and its highest level was found in the oil from CEO3. On the other hand, linalyl acetate, which was the most abundant in CEO1, decreased significantly upon ripening.

As regards a comparison with literature, it must be highlighted that the available data were obtained by analyzing essential oil from ripe Chinotto [72]. CEO3 contains limonene in the same amount, while the other components are present in different level. Interestingly, linalool and linalyl acetate were reported to be absent or present in low amount. It can be speculated that these variations can be due to different genetic origin or pedoclimatic conditions.

Table 2.18 Volatile components in CEOs

No.	Constituent	Peak Area (%)			
		CEO1	CEO2	CEO3	Lit.[2]
1	β-Pinene	Tr	0.5	0.5	19.3
2	Limonene	26.9	54.3	48.7	48.8
3	Sabinene	0.2	0.1	0.3	3.2
4	Myrcene	0.1	0.6	1.2	0.9
5	γ-Terpinene	Tr	3.7	0.4	7.8
6	*p*-Cymene	-	-	-	6.5
7	Linalool	19.6	12.1	32.4	0.5
8	Neral	0.2	0.3	0.5	1.0
9	Linalyl acetate	47.5	22.9	12.0	-
10	Geranial	0.9	0.5	0.8	1.4
11	Geranyl acetate	1.0	0.3	0.2	1.1
12	β-Caryophyllene	0.3	0.4	0.1	-
Total identified		96.7	95.9	97.5	98.9

The antioxidant properties of Chinotto essential oils (CEOs) were investigated by evaluating radical scavenging activity against a stable radical (DPPH or ABTS). In both cases, Trolox, a known antioxidant, was used as reference compound to build a calibration curve, and the results were therefore expressed as μmols of Trolox Equivalents (TE) / g of oil (Table 2.19).

Table 2.19 Radical scavenging activity of CEOs

	Ripening Stage	Scavenging activity [a]	
		DPPH	ABTS
CEO1	Unripe	6.1 ± 0.9	11.2 ± 0.07
CEO2	Semiripe	7.8 ± 0.2	10.8 ± 0.3
CEO3	Ripe	8.1 ± 0.4	9.4 ± 0.1

[a] μmols of TE / g of oil

It is interesting to note that the radical scavenging activity of CEOs increased with ripening in the case of DPPH, while it decreased in the case of ABTS.

Chinotto essential oils were also evaluated for their ability to reduce the release of nitric oxide (NO) in RAW264,7 macrophages stimulated by 0.5 µg/mL lipopolysaccharide (LPS) and incubated for 48h in the presence (or absence, in the case of the control) of increasing concentrations (1, 10 and 100 µg/mL) of CEOs. NO production has been evaluated by Griess method, by measuring nitrites in the supernatants. Essential oil from half-ripe fruits (CEO2) reduced the production of NO in a concentration-dependent way and it already effective at the concentration of 10 µg/mL, while an inhibition >50% has been observed at 100 µg/mL. On the other hand, CEO1 and CEO3 resulted ineffective in the concentration range investigated (Figure 2.12).

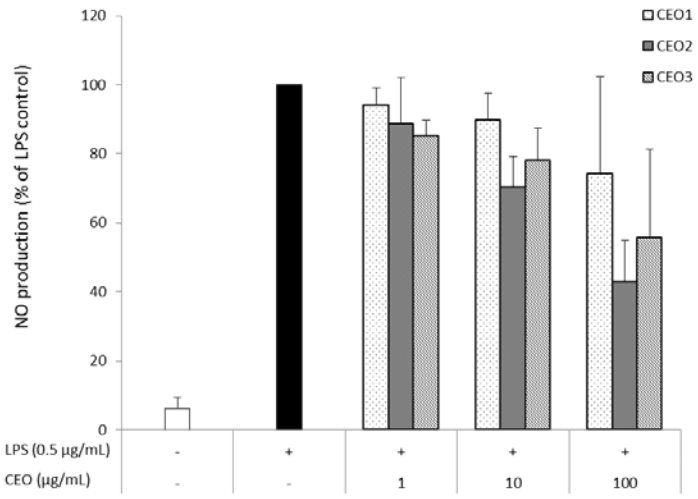


Figure 2.12 Effect of CEOs on NO production

Meanwhile, the effect of the same concentrations of CEOs has been evaluated on cell proliferation by means of XTT analysis. No cytotoxic effects were observed (Figure 2.13).

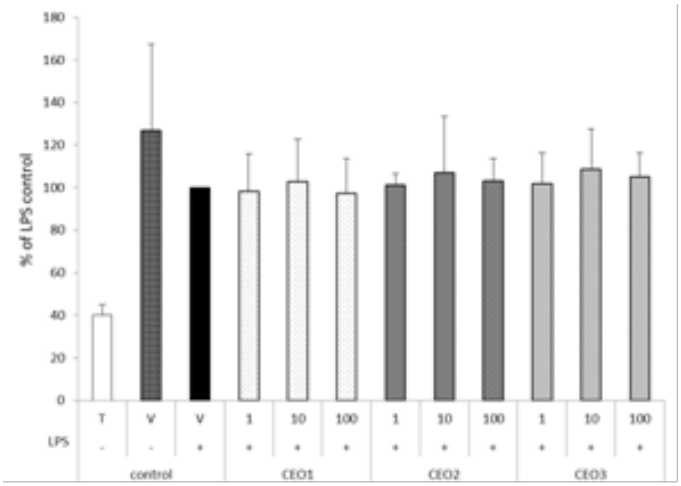
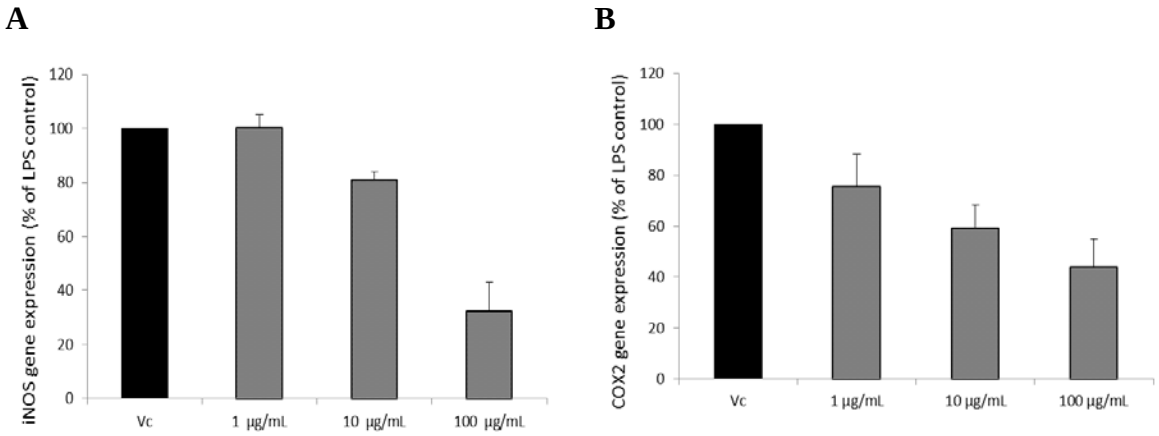


Fig. 2.13 Results of XTT analysis

Using quantitative RT-PCR, we investigated the possibility that the inhibition could occur at a transcriptional level. Indeed LPS-induced up-regulation of gene expression of inducible NO synthase (iNOS) resulted inhibited by CEO2 (Figure 2.14, panel **A**). Additionally, we found that the expression of cyclooxygenase-2 (COX-2) and of the inflammatory cytokines interleukin-1β (IL1β), interleukin-6 (IL6) and chemokine monocyte chemotactic protein-1 (MCP-1) was also reduced by CEO2 (Figure 2.14).



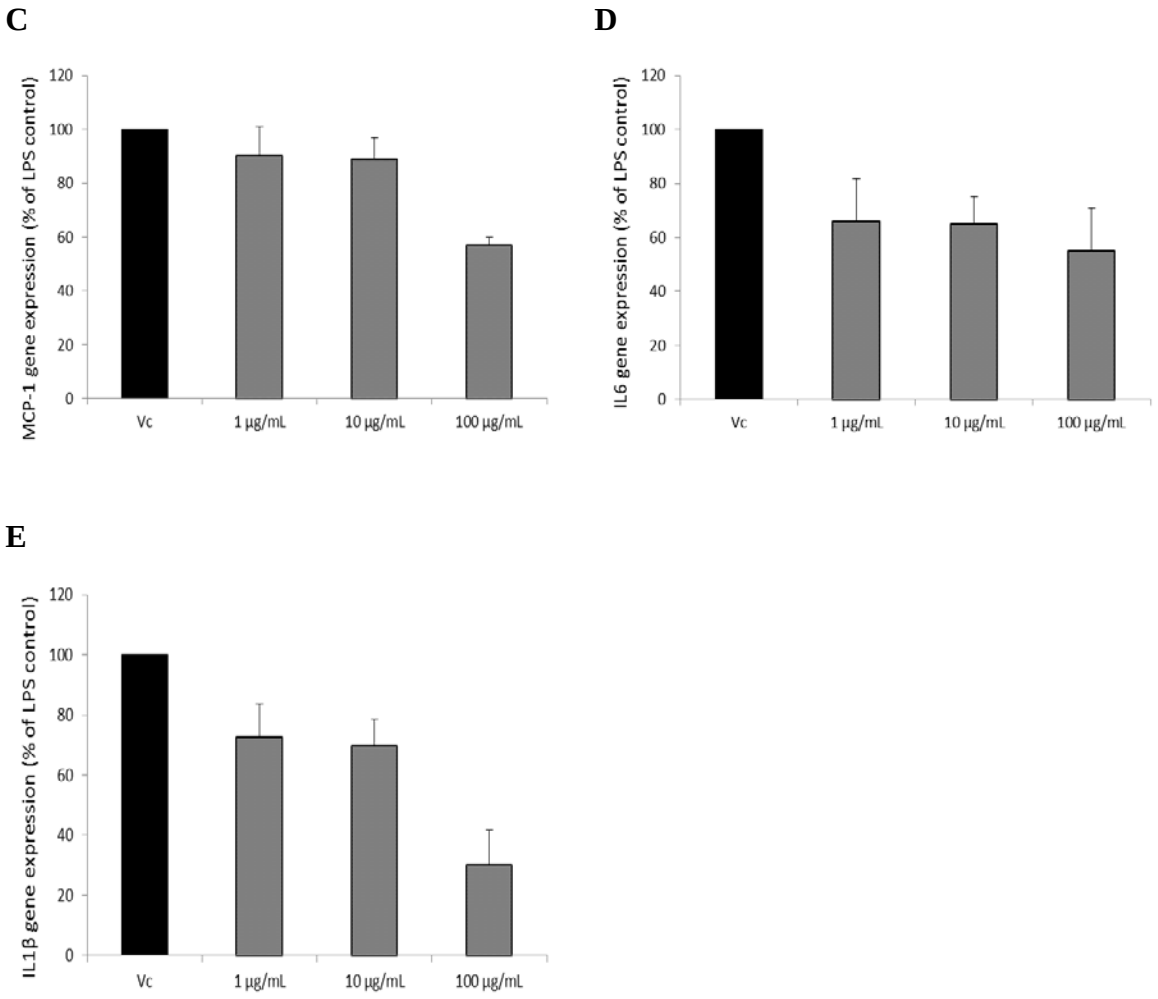


Fig. 2.14 Inhibition of gene expression up-regulation

CHAPTER 3

Experimental Procedures

3.1 Instruments

- HPLC Shimadzu LC-20AB with UV-vis detector SPD-20 and autosampler SIL-20A HT
- GC-MS Shimadzu QP-2010
- FT-IR spectrometer Perkin Elmer Paragon 1000 PC
- UV-vis, Jasco, V-550
- Lyophilizer Telstar LyoQuest
- Centrifuge Universal 320 Hettich Zentrigugen
- Scanning electron microscopy

3.2 Reagents and Standards

Petroleum ether, hexane, benzene, DMSO (ACS grade), methanol, dimethylformamide (DMF), acetonitrile (HPLC grade), ethanol, Acetone, sodium carbonate (Na_2CO_3), HCl, citric acid, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), potassium carbonate (K_2CO_3) anhydrous sodium sulphate (Na_2SO_4), were purchased from Carlo Erba Reagenti (Milan, Italy). The methyl esters of miristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2), linolenic (C18:3), arachidic acids (C20:0), oleic acid (C20:1), behenic acids (C22:0), were obtained from Sigma-Aldrich Co. (Milan, Italy). Acetyl chloride, 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulfate ($K_2S_2O_8$), potassium bromide (KBr), the Folin-Ciocalteu reagent, trolox (6-hydroxy-2,5,7,8-tetrametilcromano-2-carboxylic acid), standards of β-1,3-glucan, naringin and neohesperidin were also purchased from Sigma-Aldrich.

3.3 Plant materials

C. myrtifolia Raf. (chinotto) which were used in this study originate from Calabria and Sicily, Italy. Fruits of chinotto were collected at four different stages of ripening: unripe (harvested in October 2012), semiripe (November 2012), ripe (the first week of December 2012) and Overripe only for chinotto from Calabria which harvested on the end of December 2012. The seeds were separated from the pulp. Both of the pulps and the seeds were frozen at -20 ^{0}C before used. The Information about the sample name was shown in table 3.1.

Table 3.1 The Information of sample code

Sample Code		Degree of maturation	Regions
Pulps	Seeds		
PC02	**SC02**	Unripe	Calabria
PC03	**SC03**	Semiripe	Calabria
PC04	**SC04**	Ripe	Calabria
PC05	**SC05**	Semiripe	Sicily
PC06	**SC06**	Ripe	Sicily
PC07	**SC07**	Overripe	Calabria

All frozen chinotto pulps were dried using freeze-drying. The seeds were peeled from their seed coat. After that, the seeds were dried in oven at T = 120 °C until constant weight (t = 30 minutes). Both dried pulps and seeds were ground into powder. The pulp powder samples were stored at -20 °C prior to analysis.

3.4 Oil content of seeds

The dried seed flour (5 g) was extracted with hexane (150 mL, boiling point 69 °C) using a Soxhlet apparatus at 90 °C for 24 h. The extraction of the seed residue was repeated twice using hexane as the solvent (150 mL, boiling point= 69 °C) at 90 °C for 2 h and petroleum ether (150 mL, boiling range 30-60 °C) at 80 °C for 2 hours. The extracts were collected, the solvent was evaporated in a rotary evaporator at 35 °C to dryness, and the residue was dried under vacuum. The oil content was expressed on a dry seed weight flour. Data are reported as a mean value ± standard deviation (SD) of three samples. Oil were transferred to brown airtight containers, flushed with nitrogen and stored at -20 °C until analysis.

3.5 Fatty acid composition

The composition of fatty acid from oil of chinotto seeds was determined by gas chromatography (HRGC) after transesterification. All samples were analysed in triplicate.

3.5.1 Direct transesterification

The fatty acid composition was determined in triplicate by gas chromatography (GC) of the oils after transesterification. Fatty acid methyl esters (FAMEs) were prepared from the oils using acetyl chloride in MeOH and benzene [75]. 100 mg of dried oil were precisely weighed in a

borosilicate glass tubes. Three samples were prepared of each species (sample 1,2 and 3). Then, 1,8 mL of methanol dry, 400 μl of benzene dry and 200 μl of acetyl chloride were slowly added to each sample. The tube was tightly closed with teflon-lined caps. After stirring at 100 °C for 1 h, the mixture was cooled to room temperature and 5 mL of a 6% K_2CO_3 solution were slowly added with stirring to stop the reaction and neutralize the mixture. The esters were extracted with hexane. The supernatants containing the fatty acid methyl ester were combined, dried over Na2SO4, filtrated and evaporated to dryness. The residual oil was transferred to a vial under nitrogen gas. The dried extracts were dissolved in hexane and injected into GC immediately.

3.5.2 HRGC-FID analysis

GLC analyses were carried out on a Shimadzu GC-2010 system equipped with an AOC-20i auto sampler, split/splitless injector, and a FID detector (Shimadzu, Milan, Italy), and were performed at the following experimental conditions: the column used was a fused-silica capillary column (Supelcowax^{TM} 10) (30 m x 0.32 mm id × 0.25 μm); the oven temperature was programmed from an initial temperature of 80 °C (5 min hold), raising to 230 °C at a rate of 3.0 °C/min; the injection volume was 1.0 μl, in the split mode (17:1). Helium was used as carrier gas with the linear rate 30.0 cm/min. The detector temperature was set at 250 °C. The hydrogen flow rate was 50.0 mL/min; the air flow rate was 400 mL/ min; the make-up flow rate (N_2/Air) was 50 mL/min. The pressure injector was 101,6 kPa. Fatty acids (FA) were identified by comparison of the retention times observed with those of the commercial fatty acid methyl esters (FAMEs). Values in each sample are mean ± SD of triplicate in GC. Each value is expressed as mg/g of extracted oil.

3.6 Extraction of polyphenols

3.6.1 Defatted seed residues

Defatted seed flour (3 g) of each sample was extracted three times for 16 h at room temperature by stirring with methanol (10 mL solvent/g of flour). Between extractions, the samples were centrifuged at 5000 rpm for 10 min. The combined supernatants were collected, filtered through Whatman 0,45 μm PVDF syringe filter and evaporated to dryness, and the residue was dried under vacuum and ready to be used for analysis of antioxidant and of total phenolic content. This extraction produced yellow solid fraction.

After methanol extraction, the residual of defatted seed flour was re-extracted three times for 16 h at room temperature by stirring with dimethylformamide/DMF (10 mL solvent/g of flour). Between extractions, the samples were centrifuged at 5000 rpm for 10 min. The combined supernatants were collected, filtered through Whatman 0,45 μm PVDF syringe filter, and the residue was dried under vacuum while boiled at temperature over 80^0 C. This extraction produced yellow liquid fraction.

3.6.2 Pulp powder

The pulp powder (5 g) of each sample was extracted three times for 3 h at 0 ^{0}C by stirring with methanol (5 mL solvent/g of flour). Between extractions, the samples were centrifuged at 5000 rpm for 15 min. The combined supernatants were collected, evaporated to dryness, filtered through Whatman 0.45 μm PVDF syringe filter, and the residue was dried under vacuum and ready to be used for analysis of antioxidant and of total phenolic content. This extraction produced yellow solid fraction [7].

The second method was using DMF. Five grams of sample was extracted three times for 3 h at 0^0C by stirring with dimethylformamide/DMF (5 mL solvent/g of flour). Between extractions, the samples were centrifuged at 5000 rpm for 10 min. The combined supernatants were collected, filtered through Whatman 0.45 μm PVDF syringe filter, and the residue was dried under vacuum to give brown oil fraction [8].

3.7 Determination of antioxidant activity

The extracts of polyphenols were subjected to spectrophotometric analysis for the following determinations:

• Activities of radical scavenging through the essay with the radical DPPH.

• Activities radical scavenging through the ABTS method.

3.7.1 DPPH assay

The free-radical scavenging activity of the extracts was evaluated using the DPPH method [9]. 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical, and when it reacts with a radical scavenger, its maximum absorbance at 517 nm fades rapidly. The antioxidant effect is proportional to the disappearance of DPPH in test samples.

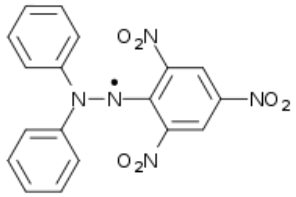


Fig. 3.1 Chemical structure of DPPH

The free radical scavenging activity against DPPH was determined at four different concentrations. Thus, MeOH solutions containing known amounts of the extract (10 mg/ml MeOH; 5 mg/ml MeOH; 2.5 mg/ml MeOH; 1 mg/ml MeOH) and DPPH 1 mM were prepared, and the colorimetric decrease in absorbance of DPPH was measured at 517 nm.

Furthermore, 100 μL of each extract solution was mixed with 100 μL of the DPPH solution, and the final volume adjusted to 3 mL by the addition of the necessary amount of MeOH. In this way, four different solutions with extract concetration of 0.33, 0.17, 0.08, 0.03 μg of extract per mL of solution were obatined for each extract. The mixtures were shaken vigorously and left standing at room temperature in the dark for 30 min. The absorbance of each sample was then measured against blank (the DPPH solution obtained by diluting 100 μL of the DPPH standard solution with MeOH to give a final volume of 3 mL) at 517 nm using a UV-vis spectrophotometer (Ultrospec 2100 pro). Experiments were carried out in triplicate, and trolox was used as the positive reference antioxidant.The % of scavenging activity on DPPH was calculated according to the formula:

DPPH scavenging ability (%) = [(Absorbance517nm of control-Absorbance517nm of sample) /Absorbance517nm of control] x 100 [76].

3.7.2 ABTS Assay

The antioxidant activity of the biophenolic extracts in chinotto pulps and seeds were also determined by spectrophotometer using the 2,2’ azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)($ABTS^{\bullet+}$) radical cation .

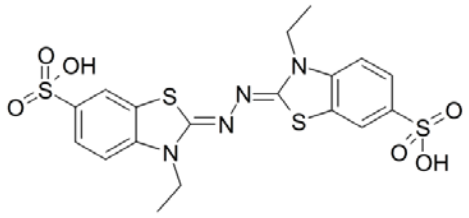


Fig. 3.2 Chemical structure of ABTS

The ABTS is generated by reacting a strong oxidizing agent, in this case potassium persulfate ($K_2S_2O_8$), with the ABTS salt and resulting of blue-green ABTS radical coloured solution. This radical cation ABTS $^{\bullet+}$ has an absorption peak at 734 nm. Antioxidant compounds that are capable of transferring a hydrogen atom or an electron to the radical cation, cause a discoloration of the solution. After a predetermined incubation time, the mix solution will measure in the UV-vis spectrophotometer at 734 nm. The decreasing in absorbance is proportional to the antioxidant capacity of the sample [77].

This assay was conducted using two options: the first was prepared by dissolving 0.0548 g of ABTS in 50 ml of H_2O (2 mM); the second was prepared by dissolving 0.189 g of $K_2S_2O_8$ in 10 ml of H_2O (70 mM). The mono cation radical ABTS $^{\bullet+}$ was produced by the mixing 500 µL of $K_2S_2O_8$solution and 50 ml of ABTS solution. The mixture of blue-green colourwas obtained by incubation the mixture for 16 hours in the dark and at room temperature. After incubation, the solution was diluted in methanol with the ratio 1:50 (the mixture/MeOH), to get the new mixture of MeOH-ABTS$^{\bullet+}$ with the absorbance between 0.7 and 0.73 at a wavelength of 734 nm.

The solution obtained was used to determine the antioxidant capacity of the biophenolic compounds contain in seeds and pulp of chinotto. For this purpose, four different amounts of extract sample were prepared and diluted in DMSO (10 mg/ml, 5 mg/ml, 2,5 mg/ml and 1 mg/ml of DMSO).

One ml of the diluted solution of ABTS$^{\bullet+}$ were added to 10 µL of sample solution; the sample was stirred for 5 minutes in the dark and immediately subjected to UV-vis spectrophotometer at 734 nm. The absorbance of each sample was measured in reference to blanco (methanol) and to a control (solution obtained by diluting 1 ml of solution ABTS $^{\bullet+}$ and 10µL of methanol).

The antioxidative activity of the tested sample was calculated by determining the decrease in absorbance at different concentration by using the following equation:

$$\% I = (A1-A0) / A0$$

It was expressed as percentage inhibition (% I). A0 was the absorbance of ABTS • + and A1 was the absorbance of the sample at 734 nm measured after 5 minutes incubation. For each concentration, three analysis were applied and trolox was used as the positive reference antioxidant.

3.8 Determination of total phenolic content (TPC)

The total phenolic content of each fraction was determined using the Folin-Ciocalteu method with some modification. First,100 μg sample of methanol and DMF extracts was diluted with DMSO up to 1 mL. Furthermore, 100 μL was taken from those extract solutions, then 1 mL of diluited (1:10) Folin-Ciocalteu reagent, 800 μL of 10% Na_2CO_3 was added and the final volume was made up to 5.0 mL with distilled water. After the mixture was left to stand for 2 h at room temperature, the absorbance at 760 nm was measured by using an Ultrospec 2100 pro UV-vis spectrophotometer. The results of total phenolic content were estimated using a standard curve prepared using gallic acid and expressed as mg of gallic acid equivalents per gram of extract

3.9 HPLC Analysis

Identification and quantification of narigin and neohesperidin were performed using the tool HPLC Shimadzu LC-20AB, equipped with two pumps SCL-10 AVP-detector SPD-20A UV-vis. Separation of each compound was performed using a Supelco Discovery C18 column (25 cm x 4.6 mm x 5 μM) at a wavelength of 278 nm and 325 nm. The injection loop was 20 μl, and the flow rate was 1.0 ml/min. The mobile phase consisted of H_2O (phase A) and acetonitrile (phase B), programmed as follows: 0 min 5% B, 0 to 15 min 20% B, 15 to 20 min 30% B, 20 to 35 min 100% B, 35 to 40 min 100% B, 40 to 45 min 5% B, 45 to 55 minutes 5% B, Stop 56 min. The analysis of each extract was tested three times. The quantitative determination was conducted by the method of the external standard, constructing calibration curves for each compound identified in the extracts.

3.10 Pectin extraction under different pH

The extraction of pectin was carried out by the method of Lima MS., *et a*l. (2009) [68], with some modifications. Five grams of each sample of chinotto seeds and pulps were added 40 ml of

distilled water (in a ratio 1: 8 m/v). The pH was brought to 2.2 with a 10% solution of citric acid. The suspension obtained was boiled for 30 minutes, cooled and filtered. The filtrate was used as a mother fraction A, which has been divided into three parts in order to obtain the three main fractions: A, A^- and A^+.

One third of the solution was added with 10% HCl to bring the pH up to 1.0. It was then heated to boiled for 30 minutes, cooled, and neutralized to pH 7.0 to obtain fraction A^- using 10% $NaHCO_3$. The other one third portion of the mother fraction A was added with 10% NaOH to reach the pH at 12. This was kept under stirring for 30 minutes at 20°C and then neutralized by addition a solution of citric acid, obtaining the A^+ fraction.

The three fractions were added with 95% ethanol to isolate the pectins by precipitation, and then centrifuged at 5000 g/20 min. The supernatant was removed and the precipitate of each fraction was washed with 99% ethanol to remove the sugars and subsequently washed with 99% acetone to remove pigments. The solid obtained were dried under vacuum and subjected to IR spectroscopic analysis.

3.10.1 Quantifying the degree of methoxylation and yield of pectins

The samples were homogenized with KBr (5:1 salt/sample) to produce KBr pellets which was sent for analysis using an infrared spectrometer. After analysis of each spectrum using FT-IR through identification of the main 1631 and 1741 cm^{-1} bands, the DM was obtained using the equation: [A1741 cm^{-1}/(A1631 cm^{-1} + A1741 cm^{-1})] in accordance with Manrique and Lajolo (2002) [78]. The yield pectin (%) was determined after dried under vacuum by weighing residue. All analyses were performed in triplicate.

3.11 Isolation of β-glucans

The method of Wood et al. (1978) [79] with minor changes was used for isolating β-glucan from pulps and seeds of chinotto. Five grams of flour samples were subjected to extraction with acetone, methanol and 70% aqueous ethanol in order to remove the oils. In order to precipitate any β-glucan presents, the residual material was then suspended in 50 ml of 20% sodium carbonate with agitation for 30 minutes. The solution was centrifuged at 5000 rpm/30 min. The residue was removed, and the pH of the supernatant was adjusted to 4.5 with HCl 2 mol L^{-1} , centrifuged at 5000 rpm/30 min to separate precipitated proteins, which were discarded. An

equal volume of ethanol was added to the supernatant to precipitate the β-glucans. After 24 hours at 4 ^{0}C, the solution was centrifuged at 3780 rpm/10 min. The precipitate was re-suspended in ethanol, filtered, rinsed with ethanol, dried under vacuum and subjected to IR spectroscopic analysis using Kbr method.

3.12 Infrared spectroscopy

Infrared spectroscopy was recorded on aPerkin Elmer Paragon 1000 PC instrument, model FT/IR-4200typeA, in the region 4000-400 cm^{-1}, and at a resolution of 4 cm^{-1}. The method used for the analysis of pectins and β-glucans was a FT-IR in KBr solid. The KBr was pulverized with a pestle in an agate mortar, and the small amount of samplewas added (5:1 salt/sample). With the resulting powder mixture, a tablet was made by compression with a pressure of 6 tons. Theinfrared transmittance of the resulting pellet was analyzed by FTIR.

Pectin from pulp

Fraction A

IR (KBr): v/cm^{-1} 3415 (s), 2933 (w). 1741 (m), 1631 (m), 1384 (s), 1098 (m), 917 (w), 620 (w)

Fraction A-

IR (KBr): v/cm^{-1} 3412 (s), 2967 (w), 1728 (w), 1613 (s), 1412 (s), 1334 (w), 1016 (s), 641(w)

Fraction A+

IR (KBr): v/cm^{-1} 3417 (s), 2957 (w), 1714 (w), 1614 (s)

Pectin from seed

Fraction A

IR (KBr): v/cm^{-1}3448 (s), 2965 (w). 1654 (m), 11534 (m), 1434 (w)

Fraction A-

IR (KBr): v/cm^{-1}3436 (s, br), 2972 (w), 1674 (w), 1607 (s), 1476 (m)

Fraction A+

IR (KBr): v/cm^{-1} 3437 (s, br), 2965 (w), 1682 (w), 1614 (s)

β-glucan from standard

IR (KBr): v/cm^{-1}3398 (s, br), 2926 (m), 1640 (m), 1047 (s), 887 (w)

β-glucan from pulp

IR (KBr): v/cm^{-1}3406 (s, br), 2920 (w), 1641 (m), 1070 (m), 916 (w)

β-glucan from seed

IR (KBr): v/cm^{-1}3416 (s), 2924 (w), 1639 (m), 1073 (m), 993 (w)

3.13 Analysis of Scanning electron microscopy

The morphological analysis of the sample of β-glucans was performedby a Leica Leo 420 scanning microscope (SEM).The sample of β-glucans were fixed on microscopestubs and they were gold coated. The electro-optical properties ofthe samples were measured at room temperature.The experimental set up for the electro-optical measurements was made up of a He-Ne Laser(632.8 nm), as light source, with a power of 2mW. The light beam went through the chopper rotating disk, and enter in a diaphragmable to eliminate reflections. Successively, the ray was directed in abeam expander in order to correct the intrinsic laser divergence, obtaining a collimate beam, and went through a second diaphragm,which reduces the dimension of laser beam incident on the sample. Transmitted light was collected by a detector, converted in an electrical signal and sent to a lock-in-amplifier, and controlled by the reference chopper signal.

The output signal was sent to a computer and recorded. The applied voltage is controlled by a Digital Analogical Converter (DAC). The measurements were performed using an alternating signal at 1 kHz.

3.14 Cell culture

RAW264.7 macrophages (murine origin) were grown and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, streptomycin and penicillin at 37 °C in a 5% CO2 humidified air atmosphere. Effects of the test Chinotto essential oils (CEOs) on the viability of the RAW264.7 cells were assessed using an XTT Cell Proliferation Kit II (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's instructions. Briefly, cells were first incubated with the test compound(s). Thereafter, the tetrazolium salt (ZTT) assay was performed, where the cells' ability to metabolise XTT to formazan was a measure for cell viability. Conditions were considered toxic if metabolic activity to form formazan was decreased by .20 %. As a negative control, Triton X100 was added to the cells, yielding total cell lysis.

3.15 Effects of CEOs on nitric oxide release and on gene expression

RAW264.7 macrophages (2.5 x 105 cells/ml) were seeded into ninety-six-well cell culture plates for nitrite, and cell viability measurements, or into six-well plates (5 x 105 cells/ml) for the analysis of mRNA expression, and incubated overnight. Adherent cells were incubated with the test CEOs in combination with LPS (0.5 mg/ml). Chloroform was used as the solvent (final solvent concentration never exceeded 0.1%, v/v). Incubation times were selected based on the specific properties of different inflammatory mediators. After incubating for 48 h, nitrite accumulated in the culture medium was measured as an indicator of NO production using the Griess method REF. Briefly, 100 ml of the cell culture medium were mixed with 100 ml of Griess reagents and incubated at room temperature for 10 min. Absorbance was measured at 540nm using an ELISA plate reader [80].

3.16 RNA purification and quantitative reverse transcription real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Breda, The Netherlands). RNA (1 mg/sample) was reverse transcribed to give complementary DNA using the reverse transcription system from Promega (Leiden, The Netherlands). Complementary DNA was amplified by PCR using platinum Taq DNA polymerase (Invitrogen) and SYBR green (Molecular Probes, Leiden, The Netherlands) on an iCycler apparatus (Bio-Rad, Veenendaal, The Netherlands). The following primer pairs were used for amplification of iNOS: 50-GTT-CTC-AGC-CCA-ACA-ATA-CAA-GA-30 (forward) e 50-GTG-GAC-GGG-TCG-ATG-TCA-C-30 (reverse); COX-2: 50-GGAGAGACTATCAAGATAGT-30 (forward) e 50-ATGGTCAGTAGACTTTTACA-30 (reverse); MCP-1: 50-CCC-AAT-GAG-TAG-GCT-GGA-GA-30 (forward) e 50-TCTGGA-CCC-ATT-CCT-TCT-TG-30 (reverse); IL-1β: 50-TGCAGAGTTCCCCAACTGGTACATC-30 (forward) e 50-GTGCTGCCTAATGTCCCCTTGAATC-30 (reverse).

Samples were analysed in duplicate, and mRNA expression levels of the different genes were normalised to RPS27A2. Primer pairs for RPS27A2 were 50-GGT-TGA-ACC-CTC-GGA-CAC-TA-30 (forward) and 50-GCC-ATC-TTC-CAG-CTG-CTT-AC-30 (reverse).

3.17 Statistical analysis

All experiments in RAW264.7 macrophages were performed in duplicate in at least three independent experiments. Data from all experiments are expressed as percentage of the LPS-

treated controls (set at 100 %). Data are presented as means ± standard deviation. Statistical differences between treatments and controls were evaluated by one-way ANOVA followed by Bonferroni's post hoc test. A P value <0.05 was considered as statistically significant.

CONCLUSIONS

The most represented fatty acids in seed oils of chinotto, both in Calabria and Sicily seeds, were linoleic, oleic and palmitic acids. The highest amount of linoleic and oleic acids belong to semiripe from Sicily.The highest amount of palmitic acid was ripe from Sicily.

The DMF and methanolic extracts from seeds and pulps were examined for their free radical scavenging activities using DPPH and ABTS assays. Those assays showed that pulp and seed extracts had the ability to scavenge DPPH and ABTS. Their antioxidant activities tend to increase during ripening, except methanolic extracts from Sicily seeds and DMF extracts from Calabria seeds in DPPH assay.

Total phenolic content in pulps of chinotto increased during ripening for MeOH and DMF extracts. However, total phenolic content in chinotto seeds decreased during maturation, except methanolc extracts from Calabria. The highest TPC in seed of chinotto, both MeOH and DMF extracts, was semiripe from Sicily. In pulp, Overripe Calabria had the highest value of phenolic contents both in MeOH and DMF extracts.

The values of naringin and neohesperidin in Calabria pulp increased during ripening but when they reached level of overripe, the values decreased. In Sicily pulp extracts, the amount of naringin and neohesperidin also increased during ripening except neohesperidin from DMF extracts.

The yield and degree of methoxylation (DM) of pectins were influenced by an initial extraction pH. The percentage yields of pectin from chinotto seeds increased during ripening. Otherwise, the amounts of pectin in pulps decreased during maturation. At ripe stage, chinotto seeds and pulps from Calabria provided more pectins than chinotto seeds and pulps from Sicily. Maturation affected the percentage of DM.The highest DM in seeds and pulps were obtained from ripe Sicily chinotto.

The amount of β-glucans in Calabria and Sicily seeds decreased during the maturation. Moreover, the amount of β-glucans also decreased in Calabria pulps during the maturation but increasedin Sicily pulps. The highest content of β-glucansin seed and pulp were obtained from unripe chinotto from Calabria. Seeds of chinotto contained more β-glucans than pulps of chinotto. The infrared spectrum of extracted β-glucans from pulps and seeds had the same profile.

The composition of essential oils from chinotto peels varies deeply during maturation. Limonene, linalool and linalyl acetate represent the most abundant compounds in all essential oils tested. Essential oil obtained from semiripe chinotto reduced the production and expression of inflammatory markers at 10 μg/ml.

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APPENDIX

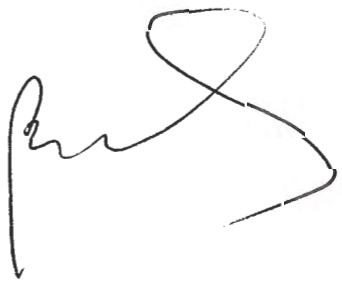
ALLEGATO A - ASTARI APRIANTINI

Il Collegio dei Docenti ha valutato l'attività di ricerca della candidata che si è sviluppata nel campo della valorizzazione dei frutti dell'area mediterranea mediante processi di estrazione, analisi e caratterizzazione, e ha preso in esame i risultati conseguiti, riportati in n° 3 lavori in preparazione.

Il Collegio ha inoltre valutato pure l'attività formativa della candidata Astari APRIANTINI che si è realizzata a seguito della assidua frequenza all'attività didattica proposta dalla Scuola di Dottorato.

Con riferimento a quanto sopra richiamato, il Collegio dei Docenti del corso di Dottorato di Ricerca in *Scienza e Tecnica – Curriculum OMPI (Organic Materials of Pharmaceutical Interest)*, giudica l'attività della candidata Astari APRIANTINI positiva e la presenta con soddisfazione al giudizio della Commissione.

Il Coordinatore OMPI



(Prof. Bartolo GABRIELE)