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PhD in

"Translational Medicine"

CYCLE XXXII

New perspective for the use of Citrus by-products in food and nutraceutical industries

Research Area: Food Science Technology (AGR-15)

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Abstract

Aim of the study: *Citrus* is the most produced tree fruit crop in the world. Almost 33% of the *Citrus* fruits are industrially processed for juice production, however, a great amount of *Citrus* wastes including peels, segment membrane and seeds are produced. Referring to the concept of circular economy, the present research project aimed to investigate a model for the reuse of *C.* × *clementina* Hort. by-products, in order to propose their utilization for the development of functional drink useful in prevention and treatment of prediabetes condition, hyperglycaemia and hyperlipidaemia.

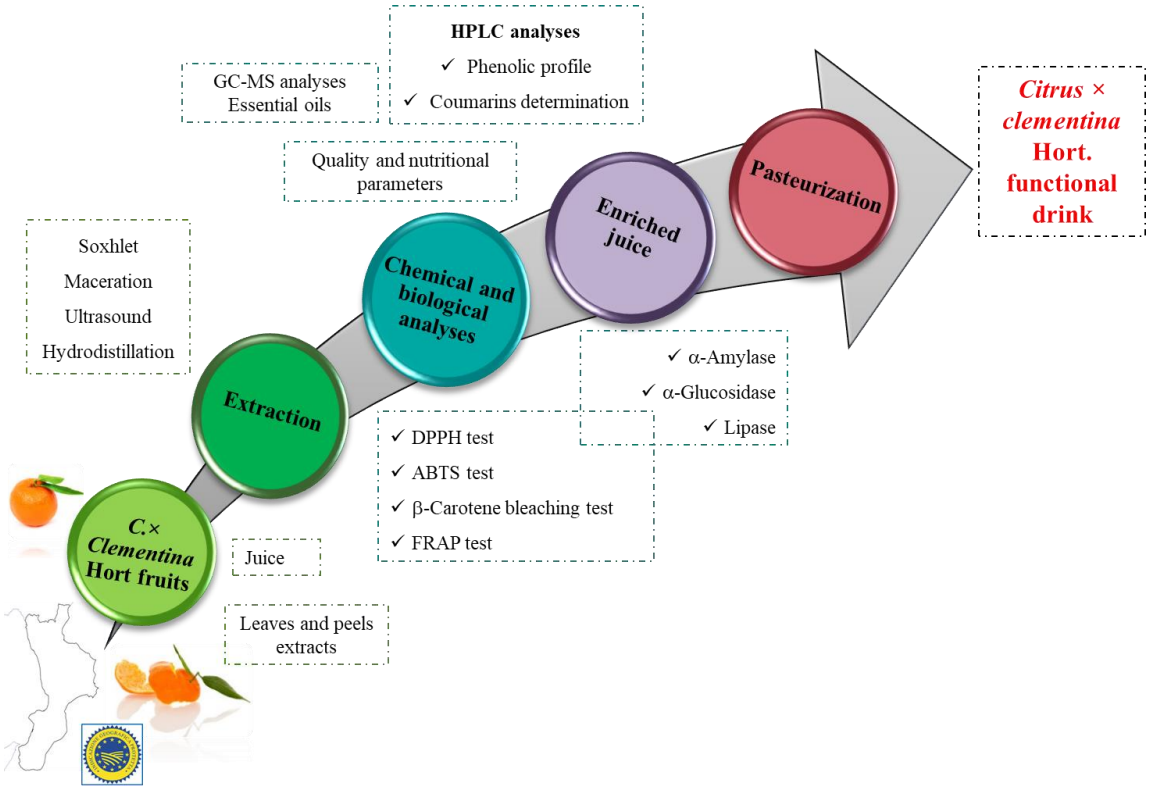
Materials and methods: The chemical profile of *C.* × *clementina* Hort. (Clementine di Calabria, PGI) juice and extracts was evaluated using HPLC-DAD and HPLC-UV. For determination of antioxidant activity four different *in vitro* tests (DPPH, ABTS, β-carotene bleaching test and FRAP), were performed. The *in vitro* hypoglycaemic potential was tested by the inhibition of α-amylase and α-glucosidase enzymes. The lipase inhibitory activity was also assessed.

Results: Juice obtained from fruits collected in Corigliano Calabro (JF) was characterized by a higher content of bioactive compounds with consequent higher bioactivity as confirmed by PCA. For this reason, JF was chosen as a matrix to be enriched with *C.* × *clementina* leaves and peel extracts. Hesperidin, tangeritin, sinensetin, and quercetin-*O*-glucoside were the most abundant constituents in all investigated samples. Considering that the content of coumarins is strictly regulated in foods, the absence of these phytochemicals represents an additional value for the potential industrial application of our samples. Among applied extraction techniques, ultrasound-assisted maceration resulted the most promising procedure to obtain extracts characterized by the highest of bioactivity in terms of antioxidant and enzymes inhibition. Indeed, leaves (from Corigliano Calabro) hydroalcoholic ultrasound-assisted maceration and peels (from Cetraro) ethanol ultrasound-assisted maceration were chosen as phytocomplex to be added to JF at different concentrations (20, 15, 10, and 5% w/v of extracts). Generally, the increase in phytochemicals content and bioactivities were significantly higher in juices enriched with leaves and leaves + peels (1:1) extracts. In order to estimate the effect of pasteurization process on our functional drink we have decided to pasteurize the 20% w/v enriched juice samples. As expected, the process decreased the bioactivity of samples in comparison to unpasteurized juice. Nevertheless, enrichment with *Citrus* by-products, especially with leaves extract, resulted in an increase of total phytochemicals content and bioactivity compared to the pasteurized control juice.

Conclusion: On the basis of obtained data regarding the interesting bioactivity of enriched juices and comforted by sensory analysis data we can propose *C.* × *clementina* by-products as a promising source of bioactive compounds useful for formulation of functional drink or foods for preventing diseases associated to oxidative stress with particular reference to hyperglycaemia and hyperlipidaemia conditions. Moreover, the high antioxidant potential of these extract allows us to propose their use as food additives able to extent the shelf-life of different foods products.

The obtained data encourage researchers to continue their work to assess the impact of functional juice on pathological models, and evaluated its bioaccessibility, bioavailability as well as possibility of hypovitaminoses due to fat reduction.

Graphical Abstract



Abbreviation

(H_2O_2)	Hydrogen peroxide
(HO_2·)	Hydroperoxyl radical
($O2^{-}$·)	Superoxide anion
(OH·)	Hydroxy radical
Acetyl-CoA	Acetyl Coenzyme A
AChE	Acetylcholinesterase Enzyme
AD	Alzheimer's disease
AFMK	N(1)-acetyl-N(2)-formyl-5-methoxykynuramine
AGEs	Advanced Glycated End Products
AMBRA	Activating Molecule in Beclin-1-Regulated Autophagy
AMK	N1-acetyl-5-methoxykynuramine
ANOVA	One-Way Analysis of Variance Test
ApoE	Apolipoprotein E
ASK1	Apoptosis Signal-Regulating Kinase 1
ATP	Adenosine Triphosphate
BC1	Soxhlet extract from Cetraro peels
BC2	Ultrasuond EtOH/H_2O from Cetraro peels
BC3	Ultrasuond EtOH extract from Corigliano Calabro peels
BC3	Ultrasuond EtOH from Cetraro peels
BC4	Maceration EtOH/H2O from Cetraro peels
BC5	Maceration EtOH from Cetraro peels
BC6	Essential oil from Cetraro peels
BHA	Butylated-Hydroxyanisole
BHT	Butylated Hydroxytoluene
BHT	Butylatedhydroxytoluene
Bm	body mass
BMI	Body Mass Index
BO1	Soxhlet extract from Corigliano Calabro peels
BO2	Ultrasuond EtOH/H_2O extract from Corigliano Calabro peels
BO4	Maceration EtOH/H2O extract from Corigliano Calabro peels
BO5	Maceration EtOH extract from Corigliano Calabro peels
BO6	Essential oil from Corigliano Calabro peels
BR1	Soxhlet extract from Rosarno peels
BR2	Ultrasuond EtOH/H_2O extract from Rosarno peels
BR3	Ultrasuond EtOH extract from Rosarno peels
BR4	Maceration EtOH/H_2O extract from Rosarno peels
BR5	Maceration EtOH extract from Rosarno peels
BR6	Essential oil extract from Rosarno peels
C*	Chroma value
CAE	Chlorogenic Acid Equivalents

cAMP	cyclic Adenosine Monophosphate
CAT	Catalase
CB1	Type 1 Cannabinoid Receptor
CB2	Type 2 Cannabinoid Receptor
CCK	Cholecystokinin
CE1	Soxhlet extract from Cetraro leaves
CE2	Ultrasuond with EtOH/H_2O extract from Cetraro leaves
CE3	Ultrasuond with EtOH extract from Cetraro leaves
CE4	Maceration EtOH extract from Cetraro leaves
CE5	Maceration EtOH/H_2O extract from Cetraro leaves
CE6	Essential Oil from Cetraro leaves
CFU	Colony-Forming Units
CO1	Soxhlet extract from Corigliano Calabro leaves
CO2	Ultrasuond EtOH/H_2O extract from Corigliano Calabro leaves
CO3	Ultrasuond with EtOH extract from Corigliano Calabro leaves
CO4	Maceration EtOH extract from Corigliano Calabro leaves
CO5	Maceration EtOH/H_2O extract from Corigliano Calabro leaves
CO6	Essential Oil extract from Corigliano Calabro leaves
COX-2	Cyclooxygenase-2
CRP	C Reactive Protein
DAG	Diacylglycerol
DIAN	*O*-dianisidine
DM	Diabetes Mellitus
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
DW	Dried Weight
EDTA	EthylenDiaminoTetracetyc Acid
EO	Essential Oils
EGP	Endogenous Glucose Production
ER	Endoplasmic Reticulum
$FADH_2$	flavin adenine dinucleotide
FFA	Free Fatty Acids
FOSHU	Food for Specified Health Uses
FPG	Fasting Plasma Glucose
FRAP	Ferric Reducing Antioxidant Power
FuFoSE	Functional Food Science in Europe
FW	Fresh Weight
GAD	Anti-Glutamic Acid Decarboxylase
GCK	Glucokinase
GC-MS	Gas-Chromatography associated with Mass Spectrometry
GDM	Gestational Diabetes Mellitus
GIP	Gastrointestinal Inhibitory Polypeptide
GLP-1	Glucagon-Like Peptide-1

GLUT	Glucose Transporters
GPx	Glutathione Peroxidase
GSTs	Glutathione-S-Transferase
HbA1c	Glycosylated Hemoglobin
HDL	High-Density Lipoprotein
HepG2	Hepatocellular Cells
HFD	High-Fat Diet
HIF-1α	Hypoxia-Inducible Factor 1-α
HL-60	Human Leukemia
HMGCR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
HNF4A	Nuclear Factor Hepatocyte-4a
HPLC	High Performance Liquid Chromatography
IAP	Intestinal Alkaline Phosphatase
IC_{50}	The concentration giving 50% inhibition
IDLs	Intermediate-Densit Lipoproteins
IFG	Impaired Fasting Glucose
IFU	International Federation of Fruit Juice Producers.
IGF	Insulin-Like Growth Factor
IGT	Impaired Glucose Tolerance
IKK β	Inhibitor of Nuclear Factor κB Kinase β
IL	Interleukin
iNOS	inducible Nitric Oxide Synthase
INSR	Insulin Receptor
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JA	Juice Sibari
JB	Juice Sibari
JC	Juice Sibari
JD	Juice Cetraro
JE	Juice Rosarno
JF	Juice Corigliano Calabro
JFA	JF + 20% CO2
JFB	JF + 15% CO2
JFC	JF + 10% CO2
JFD	JF + 5% CO2
JFE	JF + 20% BC3
JFF	JF + 15% BC3
JFG	JF + 10% BC3
JFH	JF + 5% BC3
JFI	JF + 20% CO2 and BC3 (1:1)
JFL	JF + 15% CO2 and BC3 (1:1)
JFM	JF + 10% CO2 and BC3 (1:1)

JFN	JF + 5% CO2 and BC3 (1:1)
JNK	c-Jun N-terminal kinase
JPF	JF pasteurized
JPFA	JF + 20% CO2 pasteurized
JPFE	JF + 15% BC3 pasteurized
JPFI	JF + 20% CO2 and BC3 (1:1) pasteurized
K562	Human Chronic Myelogenous Leukemia
LDL	Low Density Lipoprotein
LD_{50}	Lethal Dose, 50%
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit Of Detection
LOQ	Limit Of Quantification
LPS	Lipopolysaccharide
MAD	Malondialdehyde
MAPKs	Mitogen-Activated Protein Kinase
MetS	Metabolic syndrome
MIC	Minimum Inhibitory Concentration
MMP	Matrix Metallopeptidase
NADH	Nicotinamide adenine dinucleotide
NO	Nitric Oxide
NPC	4-Nitrophenyl Octanoate
OGTT	Oral Glucose Tolerance test
ORAC	Oxygen Radical Absorbance Capacity
PAI-1	Inhibitor of Plasminogen Activator 1
PCA	Principal Component Analysis
PD	Parkinson's Disease
PGE_2	Prostaglandin E_2
PGI	Protected Geographical Indications
PGO	Peroxidase/Glucose Oxidase
PI3K/Akt	Phosphatidylinositol 3-kinase/phosphorylated protein kinase B
PI3-K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PL	Pancreatic Lipase
PLC	Phospholipase C
PLD	Phospholipase D
POMC	Pro-Opiomelanocortin
PPARγ	Peroxisome Proliferator gamma Receptor Agonists
preDM	Prediabetes
QE	Quercetin Equivalents
RACI	Relative Antioxidant Capacity Index
REE	Resting Energy Expenditure
RNS	Nitrogen Species

RO1	Soxhlet from Rosarno leaves
RO2	Ultrasuond with EtOH/H_2O from Rosarno leaves
RO3	Ultrasuond with EtOH from Rosarno leaves
RO4	Maceration EtOH from Rosarno leaves
RO5	Maceration EtOH/H_2O from Rosarno leaves
RO6	Essential oil from Rosarno leaves
ROS	Radical Oxygen Species
S6K	Ribosomal S6 protein kinase
SGLT2	Sodium-Glucose Cotransporter-2
SOD	Superoxide Dismutase
STAT3	Signal Transducers and Activator of Transcription 3
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TA	Total Acidity
TAC	Total Antioxidant Capacity
TBARS	Thiobarbituric Acid Reactive Substances
TCC	Total Carotenoids Content
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoids Content
TGs	Triglycerides
TNF	Tumor Necrosis Factor
TNFα:	Tumor Necrosis Factor
TPC	Total Phenols Content
TPTZ	Tripyridyltriazine
TSS	Total Soluble Solids
UCP	Uncoupling Protein
VCAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low-Density Lipoproteins

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Introduction

The European Commission is undertaking a range of actions to support the move to a more circular economy. These cover the cycle from production and consumption, to waste management and the market for secondary raw materials. In a circular economy, the value of products and wastes is maintained for as long as possible, they are recovered, regenerated and reused at the end of their life (Sharma et al., 2019). The implementation of circular economy and eco-friendly initiatives creates an industrial system, which allows organisations to recycle the materials to enhance the overall sustainability. This can contribute to innovation and growth in the food and beverage industry (Bayona-Saez et al., 2017).

Food by-products are produced in large amount in the food industries, annually around the world. About 38% of food wastes is produced during food processing. Vegetable-derived processing food wastes include peelings, stems, seeds, shells, bran, trimmings residues (Helkar, 2016). Proper waste management plays a pivotal role in the growth of food industries (Kumar et al., 2017). Generally, agro-industrial wastes have been extensively used as animal feeds, fertilisers or as biofuels. However, food by-products represent a promising source of bioactive compounds, which may be re-utilized. Food industries are trying to embrace the circular economy approach by utilizing both the products and the by-products they obtain in the production chain. Furthermore, these agri-food matrices containing a wide range of bioactive phytochemicals, can be used as ingredients (food, personal and home care, pharmaceutics), food supplements (nutrition), or active compounds (pharmaceutics) (Rombaut et al., 2014).

Treated samples will be extracted with food grade hydro-alcoholic (water/ethanol) solvent mixture as environmentally friendly solvents. The extraction with hydro-alcoholic solvent will be performed by simple maceration, by ultrasound assisted extraction. The choice of the solvents takes into consideration the legislation (directive 2009/32/EC), the effectiveness towards the extraction of bioactive substances and their toxicity and environmental impact (Socaci et al., 2018). Nowadays the evolution in sample extraction and preparation has led to adopt new extraction processes that are more and more efficient, fast, reproducible and environmentally friendly. For this reason, ultrasound assisted extraction has been considered in this project for obtaining the extracts. Maceration is considered with the purpose of assessing to what extent also a simple and economical procedure available to every laboratory allows to produce extracts having interesting properties, with a particular focus on the most environmentally-friendly and less toxic solvents.

The application of innovative and *green technologies*, such as ultrasounds, will allow companies that will use them not only lower energy consumption, reduced processing times, and an improvement in the overall quality of products, but also and above, all to obtain *clean label* products characterized by better sensory qualities intended as appearance and flavour and health connotations due to the presence of bioactive extracts.

All this, in addition to representing an increase in intrinsic benefit, could be a useful tool for their valorisation through appropriate marketing strategies since the final consumer, today, is particularly attracted by the purchase of products obtained with low environmental impact technologies.

Citrus (Rutaceae) is the most produced tree fruit crop in the world. The increase in global *Citrus* production is constant in XXIth century, and annual production has reached more than 131.3 million tons (FAO, 2015).

Citrus × *clementina Hort*., a hybrid between mandarin and orange, is one of the most important crop varieties of *Citrus* in the Mediterranean area (Loizzo et al., 2018). Clementine fruits grow on different continents, and Italy represents the major European producer (Leporini et al., 2020). In Calabria (southern Italy), the cultivation of clementine is widespread due to optimal climatic conditions that have contributed to the development of food products awarded the Protected Geographical Indications (PGI) certification by the European Commission as "Clementine di Calabria" in 1997 (European Commission, 1997). Climatic and environmental conditions can cause variations in the chemical composition of the vegetable matrix. Indeed, the adaptation of many species to the natural environment that hosts them is a fundamental element for the assessment of biodiversity, understood as the chemical, genetic, and morphological variability of a plant species (Leporini et al., 2020). Hence, exogenous and endogenous factors can modify the presence/absence or abundance of a single component in the matrix. *C.* × *clementina* needs a mild climate, as constant as possible during the growing season. It is sensitive to temperature changes, especially those caused by cold winds that dry the twigs (Loizzo et al., 2018). Several literature data reported the beneficial health effects of *C.* × *clementina* fruits and *Citrus*-derived products. Some of these properties include antioxidants, hypoglycaemic, hypolipidemic, enzymatic browning inhibition, antiproliferative, neuroprotective, and antimicrobial activities, which are related to the presence of bioactive compounds including vitamin C, carotenoids, phenolics, and essential oils (Loizzo et al., 2018; Tundis et al., 2016; Sdiri et al., 2012; Camarda et al., 2007; Boudries et al., 2017; Bonesi et al., 2017; Russo et al., 2012).

Almost 33% of the *Citrus* fruits are industrially processed for juice production. However, a great amount of *Citrus* wastes including peels, segment membrane and seeds are produced. A worldwide production of 15 million tons *per* year of *Citrus* wastes was estimated. Due to the low cost and easy availability, the residues of *Citrus* fruit, discarded as waste in the environment, should be considered as potential nutraceutical source. In fact, these by-products are rich in bioactive phytochemicals, and could be recycled as value-added food supplements, which provide advantageous dietary fibers, polyphenols and other bioactive compounds. Hence, these by-products are considered a renewable resource.

The biomolecules recovered from the by-products can be used to produce functional foods and consequently offer a new opportunity for by-products reutilization. Therefore, pharmaceutical and food sectors have a common interest to obtain new natural bioactive components. The European Commission's Concerted Action on Functional Food Science

in Europe (FuFoSE), coordinated by International Life Science Institute Europe defined functional food as follows: ‘‘a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases”. The idea behind functional food is to reduce the prevalence of chronic diseases by limiting the consumption of "chemically modified" foods to give them a "healthier" appearance (Mahato et al., 2018).

Prediabetes (PreDM) is an intermediate dysregulation of glucose metabolism that exists between the normal metabolic state and the development of type 2 diabetes mellitus (T2DM) (Ferrannini et al., 2012). The prevalence of prediabetes and subsequently, the presence of insulin resistance (IR) and β-cell dysfunction, is increasing worldwide, forecasting a future increase in T2DM (Tabak et al., 2012).

Epidemiologic evidence has shown that different prediabetes criteria describe distinct populations of hyperglycaemia (Tabak et al., 2012; Kim et al., 2014). Blood glucose in the prediabetic range is correlated with many risk factors, including general and central obesity, blood pressure, triglyceride and lipoprotein concentrations (Tabak et al., 2012). Antidiabetic drugs include α-glucosidase inhibitors that reduces the rate of polysaccharide digestion from the proximal small intestine. Two large trials (Kawamori et al., 2009; Chiasson et al., 2002) support their effectiveness in the prevention of diabetes. Chiasson et al. (2002) reported in their trials, a 25% relative-risk reduction for diabetes in people with impaired glucose tolerance (IGT) who were randomly assigned to acarbose (100 mg three times a day). Almost a third of the acarbose group could not complete the trial for the appearance of gastrointestinal side-effects such as flatulence and diarrhea. Kawamori et al. (2009) investigated the inhibitor activity of voglibose, an α-glucosidase enzyme. In this trial, was observed a 40% reduction in incident diabetes risk during 48 weeks of follow-up in high-risk Japanese individuals with IGT after treatment with voglibose (0.2 mg three times a day). Although gastrointestinal side-effects were similar to those reported in previous trials, more people completed that study.

Non-antidiabetic drugs for preDM treatment include the anti-obesity drug such as Orlistat, a gastrointestinal lipase inhibitor (Tabak et al., 2012). In a post-hoc analysis of obese people (Heymsfield et al., 2000), Orlistat (120 mg 3 times a day) was associated with greater weight loss than was placebo and significantly reduced the conversion rate from IGT to diabetes in 5-year follow-up. This finding is consistent with the 4-year XENDOS (XENical in the prevention of Diabetes in Obese Subjects) trial (Torgerson et al., 2004) that reported a 37% reduction in relative risk of diabetes in obese people given Orlistat (120 mg 3 times a day). Additionally, hyperglycaemia can upregulate markers of chronic inflammation and contribute to increased reactive oxygen species (ROS) generation, which ultimately cause vascular dysfunction. Conversely, increased oxidative stress can lead to insulin resistance and impaired insulin secretion. Proper treatment of hyperglycaemia and inhibition of ROS overproduction is crucial for delaying onset of diabetes and complications (Luc et al., 2019). Prediabetes can be prevented by lifestyle interventions, of which diet is the cornerstone (Tabak et al., 2012). The efficacy of plants

and/or plant extracts in T2DM have been attributed to the diversity of active compounds with multiple mechanisms of actions that may work synergistically or potentiate the activity of each other (Graf et al., 2010; Simmons et al., 2010).

Functional food products have received enormous attention in the food market due to the growing interest of consumers in "healthy" foods. The Functional Foods Market was valued at 153600 million US$ in 2018 and will reach 260400 million US$ by the end of 2025, growing at a CAGR of 6.8% during 2019-2025.

In this context, the present PhD research project aimed to investigate a model for the reuse of *C.* × *clementina* Hort. ("Clementine di Calabria", PGI) by-products (leaves and peels), in order to propose their utilization (extracts, essential oils, and their mixtures) for the development of functional drink (enriched juice) useful in prevention and treatment of prediabetes condition, hyperglycaemia and hyperlipidaemia.

The proposed project is divided into three main sections.

1. As first objective of this project, we performed the following research tasks:
   - ✓ Sampling of *C.* × *clementina* fruits, leaves and peels from different areas.
   - ✓ Extraction of phytocomplex. Fruits were squeezed in order to obtain juice while leaves and peels were blended and extracted with different methodologies (Soxhlet apparatus, maceration, ultrasound-assisted maceration processes, and hydrodistillation).
   - ✓ Evaluation of quality and nutritional parameters of juice and investigation on chemical profile of *C.* × *clementina* juice and extracts.
2. The second strategic goal includes research on biological activity of investigated samples:
   - ✓ For *in vitro* determination of antioxidant capacity, different spectrophotometric assays namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay, β-carotene bleaching test and FRAP assay were performed.
   - ✓ Modulation of hyperglycaemia is an important tool in the management of the prediabetic patient. α-Amylase and α-glucosidase are enzymes involved in carbohydrates digestion and have been recognized as therapeutic targets for modulation of postprandial hyperglycaemia. The potential to reduce gut sugar absorption of samples was tested by inhibition of these enzymes.
   - ✓ The pancreatic lipase inhibitors are clinically approved for the treatment obesity. The potential to reduce fat absorption of samples was assessed by pancreatic lipase inhibitory assay.
   - ✓ *Pearson*'s correlation coefficient was used to describe the correlation between the biological activities and the content of components. Furthermore, the Relative Antioxidant Capacity Index (RACI) was calculated to have an overall idea of the

whole antioxidant behaviour of the different extracts, thus highlighting the most promising samples by means of multivariate analysis.

3. The third objective includes the juice selection as matrix to be enriched with *C.* × *clementina* by-products for the development of functional drink. For this purpose, the most active leaves and peels extracts were added at different concentration to the clementine juice. Additionally, we decided to pasteurize the enriched juice samples to evaluate the impact of the process on the phytochemical content and bioactivity of obtained functional products. For this purpose, the following research tasks were performed:
   - ✓ *C.* × *clementina* juice selection.
   - ✓ Selection of leaves and peels extracts to added at different concentration (20, 15, 10, and 5% w/v) to the clementine juice.
   - ✓ Evaluation of quality parameters, sensorial analysis and investigation on chemical profile of *C.* × *clementina* untreated and pasteurized enriched juice.
   - ✓ Biological activity of *C.* × *clementina* untreated and pasteurized enriched juice.
   - ✓ Multivariate analysis.

Overall, these data allow identifying and selecting the extracts having a more promising activity for the application as ingredients for the development of functional *C.* × *clementina* juice useful in the prevention of prediabetes condition, hyperglycaemia and hyperlipidaemia.

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Chapter 1:
Bioactive compounds from food by-products: new opportunities for food and nutraceutical industries

1.1 Introduction

In recent years, growing interest has been addressed in nutraceutical products that offer health benefits and an alternative to classic medicine. Nutrients, phytocomplex and dietary supplements are major constituents of this products that make them instrument to maintain health, acting against various disease and thus promoting the quality of life. The continuous growth, research developments, marketing zeal, quality assurance and regulation play a vital role in its success. Interest in nutraceuticals and functional foods is growing steadily and encourages research to identify the properties and potential applications of nutraceutical substances correlated with public interest and consumer demand.

Today, foods are not intended only to provide necessary nutrients and to satisfy hunger but, also to prevent nutrition-related diseases and improve well-being of the consumers. In this context, functional foods play a fundamental role. In fact, the principal reasons for the growth of its market are current population and health trends. In the world, populations age but life expectancies continue to grow, as does the contribution made by older individuals to the total population. Consumers can optimize the health-promoting capabilities of their diet by way of supplementation and by consuming foods that have been formulated or fortified to include health-promoting factors.

Another reason for the growing trend in functional foods is public education because consumers are more experienced in terms of nutrition through information courses. Furthermore, for consumers, "*natural*" and "*clean label*" are related to what they perceive as a healthy ingredient. For examples, the use of extracts, essential oils, chitosan, and lysozyme could replace chemical additives and contribute to the so-called "*clean label*" food, which consumers are particularly attracted to as they provide a strong picture of naturalness. Natural extracts, derived from different vegetable matrices, consisting mainly of essential oils, hydroalcoholic extracts and other derivatives, contain a wide range of secondary metabolites that can slow or inhibit the growth microorganism but also, health benefits. In fact, for scepticism about the effects of synthetic food additives on health, consumers have resorted to natural compounds, since in the '80s, the demand for these ingredients is growing. Additionally, the increasing demand on such foods can be explained by the increasing cost of healthcare, the steady increase in life expectancy, and the desire of older people for improved quality of their later years. Experts in this sector realize that besides being able to lower the cost of healthcare of the aging population, functional food might also give a commercial potential for the food industry.

1.2 Functional foods

The terms "*functional food*" was introduced by Japanese scientists in 1984. These scientists observed the relationships between nutrition, sensory gratification, fortification and modulation of physiological systems (Sirò et al., 2008). Successively, the Ministry of Health inserted regulations for approval of a specific health-related food category called FOSHU (Food for Specified Health Uses) comprehending establishment of specific health claims for this type of food.

In Eastern and Western cultures, traditional functional foods were considered a distinct class of product, identified by the "FOSHU" symbol on the food label. Conversely, in Europe and the United States, functional food meant adding functionality to an existing traditional food product and not being considered as a separate group.

The European Commission's Concerted Action on Functional Food Science in Europe (FuFoSE), coordinated by International Life Science Institute Europe defined functional food as follows: ''a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism thus either improving the general and physical conditions or/and decreasing the risk of the evolution of diseases. The amount of intake and form of the functional food should be as it is normally expected for dietary purposes (Fern, 2007). Therefore, it could not be in the form of pill or capsule just as normal food form''. The functional products are:

- ✓ ''add good to your life'' such as use of pre-probiotics which improves the regular stomach and colon functions.
- ✓ "designed to reduce an existing health risk problem" such as high cholesterol or high blood pressure.
- ✓ "products, which makes your life easier" such as lactose-free and gluten-free products.

Often the term "*functional foods*" is confused with "*nutraceuticals*". The latter, refers to almost all the bioactive components that delivers health benefits. Since a food is defined as a complex mixture of biologically active substances, its effect on health will not only be linked to the balance of these components, but also how in which they interact with or other foods components and how overall dietary intake interacts with other non-dietary risk factors for health and with the biological and genetic profile of consumers (Codoñer-Franch & Valls-Bellés, 2010).

The functional components of food (vegetables and fruits) can be effectively applied in the treatment and prevention of diseases acting simultaneously at different or identical target sites with potential physiological benefits and promotion biological activities. These components include phytochemicals, which are plant-derived, non-nutritive and biologically active chemicals. Their function in the body is to reduce the risk of certain diseases such as cancer, metabolic diseases, osteoporosis, inflammation, cardiovascular disease, degenerative diseases and neutralization of reactive oxygen species (Abuajah et al., 2015).

There are over 900 bioactive compounds found in foods. About 120 g of vegetables or fruits contain 100 different phytochemicals compounds (Abuajah et al., 2015). This new class of food products became popular in the food market due to the growing interest of consumers in "healthy" foods. Therefore, pharmaceutical and food sectors have a common interest to obtain new natural bioactive components to be used as drugs, functional food ingredients or nutraceuticals. Furthermore, these interesting bioactive compounds can be extracted from food waste and used for the development of nutraceutical and functional foods.

1.3 Revalorization of food by-products

Food waste is defined as by-product obtained from various industrial, agricultural, household and other food sector activities. Especially, food-processing industries create large quantities of by-products, which are difficult to dispose of as they have a high biological oxygen demand (Kumar et al., 2017). Indeed, disposal of wastes represents both the cost to then food processor and potential negative impact on the environment (Figure 1).

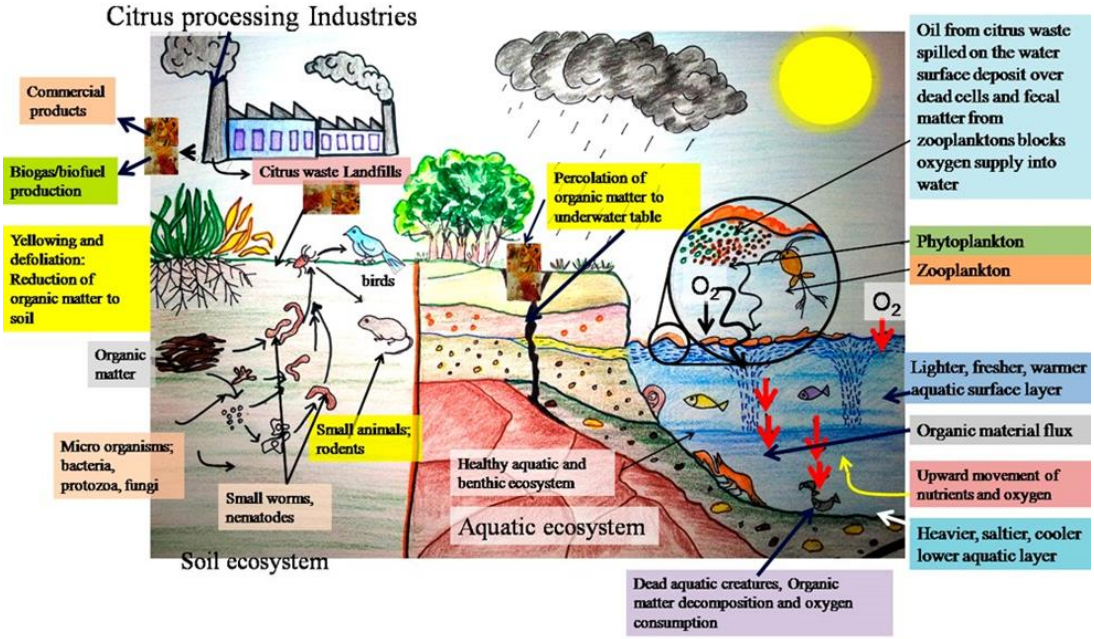


Fig. 1. Risks and potential threat to soil and aquatic ecosystems and the overall environment by the disposal of untreated *Citrus* wastes. (Adapted from Mahato et al., 2019).

The global volume of food waste is estimated to be 1.6 gigatons of "primary product equivalents," while, the total waste for the edible part of food is about 1.3 gigatons. This amount can be weighed against total agricultural production, which is about 6 gigatons per year (Growe, 2015).

Recently, much importance has been given to the recovery of bioactive compounds from different residues: peel, leaves, seeds, wood, culls, pulp, malts, hops, hulls, spent cereals (Angiolillo et al., 2015). Some of these food by-products could be revalorized as compost, feed, or transformed into biomass energy fuels and a wide variability of industrial products, such as wood-based panels, bio-fertilizers and bio-fibres (Santana-Meridas et al., 2012).

Furthermore, these agri-food matrices contain a wide range of bioactive phytochemicals with different structure and functionality that can be used as ingredients for food, food supplements or active bioactive compounds in pharmaceutical products (Rombaut et al., 2014). The techniques used to recover these biomolecules from by-products are the same as those used in extraction from raw vegetable sources. Solvent extraction from by-products can be carried out using conventional extraction processes, such as maceration, infusion and extraction of Soxhlet. However, to reduce energy consumption, the negative impact on the environment and on health, alternative extraction processes have been developed such as ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction. These new extraction technologies are considered "green extraction" and attract the interest of many researchers (Carciochi et al., 2017). Therefore, the biomolecules recovered from the by-products can be used to produce functional foods.

Bioactive compounds such as polyphenols, tocopherols, sterols, carotenoids, terpenes extracted from tomato by-products contain significant amounts of antioxidant activities (Pistocchi & Pop, 2015). These components isolated can be added to food as natural antioxidants for the formulation of functional foods or additives in to extend their shelf-life and consequently offer a new opportunity for by-products reutilization. Also, the bioactive compounds such as phenolic compounds, carotenoids, vitamin C and dietary fiber, extracted from mango peel are recognized for them contribute to lower the risk of cancer, cataracts, and neurodegenerative disease (Kumar et al., 2017).

More recently, Loizzo et al. (2019) reported that grape leaves waste should be considered an interesting ingredient for the development of functional food products. Indeed, these extracts are rich in bioactive compounds, mainly phenols that increase the defence against an excessive production of free radicals and exert a promising anti-proliferative activity against human Caucasian breast adenocarcinoma. Balzano et al. (2020) investigated the composition and bioactivity of ethanolic extract from espresso spent coffee grounds obtained by using different Arabica and Robusta coffee mixtures. This coffee waste could be a valuable source of antioxidant compounds, especially of α- and β-tocopherols, monocaffeoylquinic acids, 4-hydroxybenzoic acid, vanillin, and tyrosol. *Citrus* fruits, including oranges, grapefruits, lemons, limes, tangerines, and mandarins, are among the most widely cultivated fruits around the globe and its production is increasing every year due to rising consumer demand (Sharma al., 2017).

The global production of different types of *Citrus* fruits in the fiscal year 2016/17 for orange, tangerine/mandarin, grapefruit, lemons/limes are 50,186, 28.5, 934, and 7209 million metric tons, respectively. *Citrus* processing industries generate huge amounts of

waste every year, and citrus peel waste alone accounts for almost 50% of the wet fruit mass. Approximately than 40% of the citrus fruits produced globally are used in processing to make different commercial products, such as dehydrated products, jams, fresh juice, and flavouring agents for beverages.

As a result of processing, large amounts of after-wash waste water, peels, membranes, seeds, pulp, remain as waste products (Figure 2) (Rafiq et al., 2018). *Citrus* waste is of immense economic value as it contains an abundance of various polyphenols, flavonoids, carotenoids, essential oils, dietary fiber, sugars, and ascorbic acid. The peels, in particular, contain 15% higher content of phenolic compounds compared to the edible portions.

In food industry, *Citrus* by-products and its value-added compounds are utilized as natural additives, with the following properties: antimicrobials, antioxidants, colorants and flavouring agents (Mahato et al., 2019). This resource represents an alternative to synthetic food additives, which are associated with negative effects on human health.

Thus, new aspects concerning the use of these wastes for further exploitation on the production of food additives or supplements with high nutritional value have gained increasing interest.

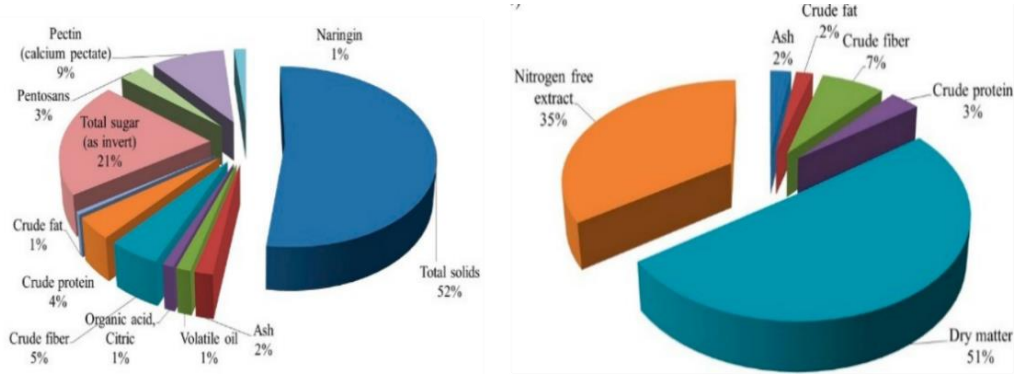


Fig. 2. (a) The composition of typical citrus waste (peel and rag); (b) the composition of dried citrus pulp. (Adapted from Mahato et al., 2019)

Citrus by-products act as protective agents for prevention of degenerative processes through their incorporation into functional foods, nutraceuticals, and cosmetics. These beneficial effects have been attributed mainly to their radical scavenging activities and consequently, inhibit the oxidation of DNA, proteins and lipids. In fact, the oxidative changes in food are responsible for the development of off-flavours by formation of compounds that result in a decrease in its sensory and nutritional quality (Kumar et al., 2017).

Most of the antioxidants currently used are synthetic and include butylated-hydroxyanisole (BHA) and butylatedhydroxytoluene (BHT). These compounds show sometimes side-effects and are implicated on development of cancerous cells in rats. For

this reason, the interest for the use of natural antioxidants has also been strengthened, also related to a greater preference of consumers for safer, healthier and less dangerous foods and ingredients than synthetic.

Extraction and isolation of *Citrus* by-products represent an alternative source for retard lipid oxidation in food and thereby improve the quality and nutritional value of food (Kang et al., 2006). The same research group investigated the ability of *Citrus* peel powder to inhibit lipid oxidation in salmon meat homogenate incubated at 37 °C. Salmon is a fatty fish and the meat is more sensitive to oxidative deterioration due to its high polyunsaturated fatty acid content. Homogenization determine a disruption of muscle cell membrane and interaction of unsaturated fatty acid with pro-oxidant substances (non-heme iron) and thereby accelerated lipid oxidation and development of rancidity. Oxidative degradation of PUFA generates a number of degradation products including malondialdehyde (MAD), which is a major component measured as an index of lipid peroxidation. Results suggest that the freeze-dried *Citrus* peel after hydroalcoholic extraction, followed by a γ-irradiation treatment, gave significant protection to lipid oxidation as indicated in 2-thiobarbituric acid reactive substances (TBARS) test.

Rafiq et al. (2018) confirmed that by-products (peels and leaves) of two *Citrus sinensis* and *C. aurantium* acted as potent antioxidants against lipid peroxidation. Additionally, these compounds also shown antimicrobial effects, playing an important role against pathogenic agents through cellular lysis. Different antimicrobial packaging systems, including lemon extracts, was used to preserve Mozzarella cheese. Results demonstrated an increase in the shelf life of all active packaged Mozzarella cheese, confirming that lemon extract may exert an inhibitory effect on the microorganisms responsible for spoilage phenomena without affecting the functional microbiota of the product. Lime and lemon peel essential oils are widely used as aroma flavour enhancers for soft and alcoholic beverages and food, while in pharmaceutical industries used as flavouring agents to mask unpleasant tastes of drugs (Kodagoda & Marapana, 2017).

Studies with fresh meat, meat products, fish, milk, dairy products, vegetables, fruit and cooked rice showed that the concentration needed to achieve a significant antibacterial effect is around 0.5-20 μL/g in foods and about 0.1-10 μL/mL in solutions for washing fruit and vegetables (Gowe, 2015). Most of these materials from *Citrus* by-products could be used as functional ingredients when designing healthy foods, especially non-digestible carbohydrates such as dietary fiber. The increase of dietary fiber in the daily diet is very important for keeping good health (Mahato et al., 2018). Therefore, for these reasons, there is an increasing interest in the consumption of fiber rich foods at daily basis. Adding fiber to frequently consumed foods such as meat, dairy products and baked goods could help reduce the current health problems associated with fiber deficiency.

Citrus wastes, especially peels, are good source of fiber. The incorporation of fibres in food processing have shown a substantial improvement not only in the health of the consumer but also in the cooking yield of the food, improving the consistency of food products. In fact, in the meat the fiber, thanks to their properties of binding to water and fat, improve the consistency reducing formulation cost (Mahato et al., 2018). Hence, these

by-products are considered a renewable resource. The idea behind functional food is to reduce the prevalence of chronic diseases by limiting the consumption of "chemically modified" foods to give a "healthier" appearance (Mahato et al., 2018). Furthermore, the extracts obtained from the *Citrus* by-products are successfully used in therapeutic formulations to provide a substantial level of protection for humans from various diseases including diabetes, obesity related to oxidative stress (Kumar et al., 2017; Mahato et al., 2018).

1.4 Oxidative stress

Oxidative stress is defined as an imbalance between the production of Radical Oxygen Species (ROS) and the antioxidant defensive mechanisms in our body.

1.4.1 The reactive oxygen species (ROS)

ROS can be divided into:

- ✓ free radicals are molecules containing one or more unpaired electrons
- ✓ non-radicals are created when two free radicals share their unpaired electrons

Production of an excess of free radicals that are not counteracted by defensive antioxidants causes oxidative damage, which is related to aging and several chronic diseases including metabolic diseases, cardiovascular diseases, neurodegenerative disorders, etc. Free radicals are generated in the body by normal metabolic functions such as cellular respiration, exposure to microbial infections involving phagocyte activation, during intensive physical activity or the action of pollutants/toxins such as cigarette smoke, alcohol, radiations, UV, pesticides, and ozone (Mahato et al., 2018). In the aerobic process that employ oxygen to oxidize carbon and hydrogen-containing biomolecules to produce chemical energy and heat, molecular oxygen is stepwise reduced to a series of intermediate species (Pistocchi & Pop, 2015).

A) $O_2 + e^- + H^+ \rightarrow H_2O^{\cdot}$

Hydroperoxyl radical ($HO_2^{\cdot}$) is the protonated form of superoxide; the protonation/deprotonation equilibrium exhibits a pK of around 4.8. Consequently, about 0.3% of any superoxide present in the cytosol of a typical cell is in the protonated form.

B) $H_2O^{\cdot} \rightarrow H^+ + O_2^{\cdot -}$

Superoxide anion ($O2^{-\cdot}$) is generated by an electronic oxygen reduction of various oxidases, such as, dihydro nicotinamide adenine dinucleotide phosphate oxidase, cyclooxygenase, xanthine oxidase, but also in the course of oxidative phosphorylation that produces ATP. Additionally, in the electron transport chain, electrons are transferred from NADH and $FADH_2$ to molecular oxygen, reducing it by generating superoxide radical anion (Pistocchi & Pop, 2015). These molecules, act as a strong reducing agent against iron complexes like cytochrome C, ferric-EDTA but also with hydrogen donors

such as ascorbate and tocopherol. The superoxide radical anion can be inactivated by enzymes belonging to the superoxide dismutase family (Drose & Brandt, 2012).

C) $O_2^{\cdot-} + 2H^+ + e^- \rightarrow H_2O_2$

Hydrogen peroxide (H_2O_2) can be generated from superoxide, in the presence of oxidase such as glucose oxidase, urate oxidase, D-amino acid oxidase. These enzymes can directly synthesize hydrogen peroxide by transferring two electrons to molecular oxygen. Hydrogen peroxide produce highly reactive radicals due to its interaction with metal ions which determine the attack on the structure of blood proteins with iron release, enzymatic inactivation and oxidation of DNA, lipids, -SH groups and keto-acids. The action of H_2O_2 can be inhibited by the catalase, which contains heme engaged in the conversion of hydrogen peroxide into water (Pistocchi & Pop, 2015).

D) $H_2O_2 + e^- \rightarrow HO^- + HO^{\cdot}$

E) $HO^{\cdot} + H^+ + e^- \rightarrow H_2O$

Hydroxy radical (OH·) represents the most aggressive radical species, responsible for the oxidative damage of most biomolecules. These hydroxyl radicals can be generated by Fenton type reactions or by water radiolysis. OH· interacts with most organic and inorganic molecules such as DNA, proteins, amino acids, lipids, metals and sugars. These reactions are characterized by a high speed, high reactivity and short duration of hydroxyl radicals involving the extraction of hydrogen, the addition and transfer of electrons (Kohen & Nyska, 2002). ROS modulate the function of all classes of biomolecules, damaging almost all substrates in the cell including lipids, proteins and DNA. Lipids are the most sensitive to oxidation and readily undergo peroxidation by OH· attack. In particular, polyunsaturated fatty acids, such as docosahexaenoic acid and arachidonic acid generate MAD and 4-hydroxynonenal.

These reactive aldehydes are recognized as markers of lipid oxidative decay and 4-hydroxynonenal can get attached to proteins and may therefore impair their function (Doorn & Peterson, 2003). In addition, ROS oxidize the proteins through side-chain oxidation, backbone fragmentation, unfolding and misfolding, resulting in activity loss. These altered proteins, subsequently interact with the amino acid side chains to generate carbonyl functions. The damage to nucleic acids occurring by ROS because they cause cross-linking of DNA-protein, wire breakage and alteration of the structure of purine and pyridine bases, resulting in DNA mutations. All amino acids are sensitive to oxidation but cysteine and methionine are the most susceptible. Many of these oxidations are reversible by the action of disulphide reductases but, *in vivo*, a series of irreversible alterations was observed, such as the formation of S-carboxymethylcysteine and S-(2-Succinyl) cysteine (Alderson et al., 2006).

Also, lysine undergoes oxidation processes ROS-mediated with formation of carbonyl derivatives such as carboxymethyl lysine (Pistocchi & Pop, 2015). Oxidative damage to

mitochondrial membranes and protein structure may in turn increase ROS generation, leading to cell death by apoptosis mechanism (Ricci et al., 2008). Apoptosis signal-regulating kinase 1 (ASK1), represents an important marker of apoptosis initiation by oxidative stress. Its activity is regulated by thioredoxin-1, an oxidoreductase that binds to the reduced form of ASK1. When thioredoxin-1 is oxidized, the ASK1 binding is inhibited, with consequent activation of the subsequent c-Jun N-terminal kinase apoptosis pathway (Pistocchi & Pop, 2015).

1.4.2 Antioxidant agents

Antioxidants, classified as endogenous and exogenous, are compounds or systems able to delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radicals. Antioxidants inhibit the action of free radicals through different mechanisms that include:

- ✓ scavenging species responsible to initiate peroxidation
- ✓ chelating metal ions and thus blocking the formation of reactive species and/or decomposition of lipid peroxides
- ✓ quenching superoxide anion preventing formation of peroxides
- ✓ breaking the auto-oxidative chain reaction
- ✓ reducing localized O_2 concentrations (Brewer, 2011).

Endogenous antioxidants

This class includes:

- ✓ superoxide dismutase that depletes superoxide radical anion. Three different types of superoxide dismutase were identified in in mammalian tissues: copper-zinc containing superoxide dismutase found in the cytosol (SOD1), manganese containing superoxide dismutase present in the mitochondrial matrix (SOD2) and extracellular superoxide dismutase (SOD3).
- ✓ catalase that depletes hydrogen peroxide to water. This enzyme is expressed in the majority of the organs, cells, and tissues and at high concentrations, in erythrocytes and in the liver.
- ✓ glutathione peroxidase, a selenium-containing enzyme, that catalyses the reduction of hydrogen peroxide, and organic hydroperoxides to water or corresponding alcohols.

These enzymatic antioxidants are called also, “the first line of defence”, while the “second line of defence” include mainly reduced thiols and low molecular-weight antioxidants. In addition, secondary antioxidants can act in synergy with primary antioxidants stabilizing primary antioxidants by creating an acidic environment, regenerating primary antioxidants by hydrogen donation, chelating pro-oxidative transition metal cations and quenching molecular oxygen (Pistocchi & Pop, 2015).

Other antioxidants are:

- ✓ Thiols are recognized as essential antioxidant buffers as interact with most of physiological oxidants. Their capacity to maintain the homeostatic intracellular

and tissue redox status is based on the thiol/disulphide redox couple such as in the case of glutathione. The latter is recognized as scavenger of hydrogen peroxide and hydroxyl anion chlorinated oxidants and found in most tissues in liver of 5-10 mM. The glutathione is soluble in water and therefore, it is able to protect water-soluble proteins, while bilirubin, another endogenous antioxidant, is lipophilic and prevents peroxidation of membrane lipids (Sedlak et al., 2009).

- ✓ Uric acid act as scavenger of reactive radicals resulting from autoxidation of hemoglobin, or peroxide generation by macrophages. In particular, uric acid inhibit singlet oxygen, peroxyl and hydroxyl radicals and protects erythrocyte membrane from lipid peroxidation (Pistocchi & Pop, 2015).
- ✓ Melatonin is an amphiphilic endogenous antioxidant that was able to scavenge hydroxyl radical, superoxide anion radical or nitric oxide and inhibits mitochondrial oxidative stress (Lowes et al., 2013). The antioxidant activity is correlated to its metabolites such as N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), with known reductive and anti-radicalic activity, downregulate pro-oxidant and proinflammatory enzymes such as inducible nitric oxide synthase and cyclooxygenase-2 and also, downregulate pro-oxidant and pro-inflammatory enzymes such as inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). In particular, AMK keeps the ability of melatonin to prime mitochondria complex I, ameliorate ATP production by limiting the electron outflow and inhibits the opening of mitochondrial permeability transition pore (Pistocchi & Pop, 2015).

Exogenous antioxidants

This class include a wide range of molecules or components found in food. A diet rich in antioxidants can bring health benefits related to the antioxidant capacity of natural products.

- ✓ Ascorbic acid is one of the most common hydro-soluble antioxidants. Food sources include *Citrus* fruits, berries, tomatoes, potatoes, broccoli, cauliflower, Brussels sprouts, red and green bell peppers, cabbage, and spinach (Hands, 1995). His L-enantiomer, vitamin C, is involved in maintenance integrity of the vascular and connective tissue, in iron absorption and collagen biosynthesis. Vitamin C acts as a neuroprotective with radical scavenging activity and is a cofactor of dopamine β-hydroxylase and thus, takes part in biosynthesis of this catecholamine. Furthermore, it plays a role in leukocyte functioning, hematopoiesis and protects membrane phospholipids from peroxidative damage (Pistocchi & Pop, 2015). In fact, this exogenous antioxidant, blocks the alkoxy, hydroxyl and superoxide anion radicals in biological systems. Vitamin C is able to inhibit reactive nitrogen species with formation of semidehydroascorbic acid and therefore, to prevent the oxidative decay of essential biomolecules. Du et al. (2012) reported that ascorbic acid act in synergism with tocopherol, in removing

lipid peroxide radicals. At the lipid-aqueous interphase, ascorbic acid reacts with membrane-bound oxidized tocopheroxyl radical reducing it, and regenerating active tocopherol, able to accomplish its antioxidant roles.

- ✓ Vitamin E (α-tocopherol). Food sources include leafy green vegetables, almonds, hazelnuts, and vegetable oils like sunflower, canola, and soybean (Hands, 1995). This vitamin act against lipid peroxidation of cell membranes and blocks the radical chain (especially $^{\cdot}O_2^-$) by forming a low-reactivity derivative unable to attack lipid substrates. Thus, vitamin E plays an important role in membrane preservation against free radical damage promoted by low-density lipoproteins. α-Tocopherol is found in plant leaves, located in the chloroplast envelope and thylakoid membranes in proximity to phospholipids (Brewer, 2011)
- ✓ Carotenoid act as scavenger of free radicals through three possible mechanisms: electron transfer, hydrogen abstraction and radical addition. Carotenoids are pigments synthesized by plants and also found in seafood and some living organisms, such as salmon, birds, and mammals, which obtain them in their diets (Mlcek & Rop, 2011). Food sources include carrot, beans green, broccoli, cabbage, sweet corn, lettuce, pepper, spinach, tomato, *Citrus* fruits and watermelon (Kopsell & Kopsell, 2010). β-Carotene is one of the most important carotenoids. This compound is able to quench singlet oxygen, possess an important role in systemic photoprotection for its antioxidant properties, protect against UV light-damage of skin, macular degeneration, cataract, and whose addition to diene structures results in endo-peroxides (Pistocchi & Pop, 2015).
- ✓ Phenolic acids are a wide group of metabolites that originate from the secondary metabolism of plants. Phenolic compounds are present in almost all foods of plant origin with particular reference to apple, banana, black plum blackberry, blueberry, cherry, *Citrus* fruits, cranberry, guava, litchi, mango, peach, papaya, pineapple, plums, prunes, raisins, raspberry, red grape, starfruit, strawberry, broccoli, brussel sprouts, cabbage, carrot, cucumber, mint, spinach, tomato, onion varieties etc. (Balasundram et al., 2006). Phenolic compounds are considered potent antioxidant for their capacity to increase catalase activity, to trap reactive oxygen species and to act as a metal chelator. Additionally, they determined the inhibition of chain lipid peroxidation, by trapping peroxyl radical and can quickly react with peroxy-nitrite (Pistocchi & Pop, 2015). The common characteristic of the flavonoids is the basic 15-carbon flavan structure arranged in 3 rings A, B, and C. The free radical-scavenging potential of natural polyphenolic compounds depend both number and location of free -OH groups on the flavonoid skeleton. Indeed, the B-ring substitution pattern is especially important to free radical-scavenging ability of flavonols. Flavonoids with multiple hydroxyl groups are more effective antioxidants than those with only one and the presence of the ortho-3,4-dihydroxy structure increases the antioxidant activity. Additionally, the steric relationship of these −OH groups and their arrangement on the ring(s) both play a role in the ability of the substance to chelate metal ions (Brewer, 2011).

1.4.3 Critical view on the antioxidant-oxidant balance

Nutritional supplements are traditionally employed for overall health and for management some health conditions, although controversies are found concerning the role of antioxidants-mediated benefits *in vivo* (Di Pierro et al., 2020). The interplay of both endogenous and exogenous antioxidants with the systemic redox system is very complex and represents an issue that is still under debate (Conti 2016).

Trials on antioxidant supplementation demonstrated differences results (Pistocchi & Pop, 2015). Discordant data are justified by:

- ✓ the type of subjects treated, with general or high-risk;
- ✓ the different supplement doses administered in nutritional amounts or higher. The antioxidative or prooxidative effects are dose dependent (Poljsak et al., 2013).
- ✓ the type of intake in single or in balanced combination. The antioxidant supplementation might have interdependency, the compounds exhibit their effectiveness when administered in mixtures, and this implies reciprocal influences (Pistocchi & Pop, 2015).

It has been confirmed that fruits and vegetables represent secure sources of antioxidant vitamin amounts lowering oxidative stress. However, it has been reported that high antioxidant supplementation can prove unsafe with change the physiological balance between ROS generation and removal. Additionally, endogenous systems and repair processes of the cell, under conditions of enhanced activation, use important energy amounts, too great to assure complete protection against oxidative decline (Poljsak et al., 2013).

The recommended daily dietary intake of vitamin C in healthy individuals is approximately 100 mg and produces plasma vitamin C levels between 60 and 100 μmol/L (Levine et al., 1999). Theoretical, concerns exist that high-dose vitamin C may exert pro-oxidant effects. By donating an electron during radical scavenging, vitamin C is converted to the ascorbate radical and after a further electron donation to dehydroascorbic acid (DHA). During this process, a more aggressive radical, superoxide is converted to the less aggressive ascorbate radical which predominantly reacts with itself, thereby dismutation to DHA and ascorbate. In addition, electrons from ascorbate can reduce copper and iron, and generate superoxide and hydrogen peroxide, H_2O_2 (Padayatty & Levine, 2016). Of the more than 20 different oxidative DNA lesions, 8-oxoguanine (8-oxogua) and its respective nucleoside 8-oxo-29-deoxyguanosine (8-oxod) appear to be the most abundant and most mutagenic. 8-Oxogua is formed by attack of guanine by hydroxyl radicals, peroxynitrite, or singlet oxygen and causes a transversion mutation after replication (Carr & Frei, 1999).

The human oxidative biomarkers data on the role of vitamin C are controversial and inconsistent. Some studies reported that treatment after vitamin C showed a vitamin C-dependent reduction in oxidative DNA damage, while some studies found no change or an increase in the levels of selected DNA lesions (Poljsak & Ionescu 2009). Previosly, Podmore et al. (1998) reported that in volunteers the supplementation with 500 mg of

vitamin C daily for 6 weeks determined a significative reduction of 8-oxogua levels, while the levels of 8-oxoade were significantly elevated. Since 8-oxoade is at least 10 times less mutagenic than 8-oxogua, the authors suggested that the overall effect of ascorbate intake is profound protective. These results were in according with a placebo-controlled study of Vojdani et al. (2000) in which increasing concentrations of vitamin C administered to humans (500mg, 1000mg and 5000mg per day, respectively) no DNA oxidation products, but a reduction of apoptosis and an increase of NK-cell cytotoxic activity, were observed. Lee et al. (1998) suggest that supplementation with antioxidants antioxidants 200 IU of vitamin E, 9 mg of β-carotene, 500 mg of vitamin C for 4 weeks, might protect smokers from oxidative damages and could reduce cancer risk or other diseases caused by free radicals associated with smoking. The results showed a reduction 8-oxoguanine level, whereas the level of 8-oxoadenine increased. Porkkala-Sarataho et al. (2000) observed that neither vitamin E nor vitamin C, nor the combination influenced the urinary excretion rate of 7-hydro-8-oxo-2-deoxyguanosine. No relationships between vitamin C intake and urinary markers of DNA damage were observed with 500 mg/day vitamin C by Cooke et al. (1998).

A metanalysis (Ashor et al., 2014) revealed that vitamin C supplementation counteracts endothelial dysfunctions, which is doubtless one of the major contributors for both the development and progression of cardiovascular diseases. This study suggested a clinical impact of supplementation only in subjects at higher cardiovascular diseases risk. Antoniades et al. (2004) found that a vitamin C supplementation of 2 g/day for 4 weeks increased forearm vasodilator response to reactive hyperaemia in patients with combined diabetes and coronary artery diseases. An interesting study by Mullan et al. (2002) showed that an oral administration of ascorbic acid (500 mg/day) for 1 month lowered blood pressure and reduced systemic arterial stiffness; conversely, Ghosh et al. (1994) in randomized controlled trials failed to prove a blood pressure-lowering effect of vitamin C supplementation.

Recently, the beneficial effects on triglyceride and HDL of vitamin C concentrations observed by Ashor et al. (2016) might be explained by the ability of vitamin C to counteract the oxidative stress-induced insulin resistance in diabetic individuals. The vitamin C induced insulin sensitivity may ameliorate the insulin resistance induced dyslipidaemia in diabetics.

Several of the studies showed a combination of effects depending on the study systems or experimental design. Even in the presence of iron, vitamin C predominantly reduced *in vivo* oxidative damage, despite its well-known pro-oxidant properties *in vitro* in buffer systems containing iron. In more complex and physiologically relevant *in vitro* systems, such as isolated or cultured cells and biological fluids an antioxidant role, or no effect of vitamin C, predominated over a pro-oxidant role (Carr & Frei, 1999).

1.4.4 Antioxidant supplement in clinical trials

The interest in using natural products in clinical trials has recently been on the increase. In 2017, the National Centre for Complementary and Integrative Health

(NCCIH) introduced new funding opportunities for natural product clinical trials through a webinar.

Oxidative stress has a key role in the pathogenesis metabolic syndrome and its risk factors, including central obesity, type 2 diabetes mellitus (T2DM), and cardiovascular disease. Antioxidant therapy has been suggested as a potential approach to blunt its development and progression (Xu et al., 2018).

Bernabé et al. (2013) analysed the effect of a *Citrus*-based juice (juice *Citrus* 95% with 5% of *Aronia melanocarpa* extract) on biomarkers of oxidative stress in patients with metabolic syndrome compared with healthy individuals. The study comprised 20 healthy subjects and 33 patients with metabolic syndrome. Eighteen patients consumed daily 300 mL of a *Citrus*-based juice during 6 months and 15 patients consumed 300 mL of a placebo beverage. The control group consumed a *Citrus*-based juice. The results showed that the consumption of *Citrus*-based juice improved the biomarkers of oxidative stress in metabolic syndrome patients with reduction of 8-hydroxydeoxyguanosine, carbonyl groups and oxidized LDL in both groups and increase of GSH/GSSH (reduced and oxidized glutathione). Additionally, the intake of 300 mL of this fruit juice produces no variations of glucidic parameters but improvement of lipidic panel, with decrease in the cholesterol, LDL-C and C-reactive peptide levels, in subjects with metabolic syndrome (n = 33) compared with healthy subjects (n = 20) in a clinical trial of 4 or 6 months (Mulero et al. 2012). The protective effects of *Citrus* were also investigated by Dallas et al. (2014). They evaluated the efficacy and safety effects of Sinetrol-XPur (polyphenolic *Citrus* dry extract) in oxidative status. In a 12-week, randomized, double-blind, placebo-controlled trial, Sinetrol-XPur was given to overweight subjects twice daily with meals in the tested group (n= 47) *versus* a placebo group (n= 48). Oxidative stress was lowered as seen by the reduction of malondialdehyde (-14.03% vs 2.76%) and the increase in superoxide dismutase and glutathione (17.38% vs 2.19% and 4.63% *vs* 2.36%, respectively). No adverse effects were observed. Kidney, liver, and lipid panels remained unchanged. The results indicated that Sinetrol-XPur supplementation is a viable option for reducing oxidative status in healthy overweight individuals.

The beneficial effects of tomato juice consummation (330 mL) on oxidative stress status on erythrocyte antioxidant enzymes, SOD, glutathione GPx and CAT, TAC, and MDA was investigated by Pourahmadi et al. (2015) in overweight and obese females (n= 32) for 20 days. Daily dietary intake, reduces oxidative stress in overweight and obese females and, may prevent from obesity related diseases and promote health. Indeed, plasma TAC and erythrocyte antioxidant enzymes increased and serum MDA decreased in the intervention group compared the control group. In a randomized, double-blind, placebo-controlled trial, 44 T2DM patients were randomly assigned to one of two groups: group A (pomegranate juice, n= 22) and group B (placebo, n= 22). At the baseline and the end of 12-week intervention, biochemical markers of oxidative stress were measured. At baseline, there were no significant differences in plasma TAC levels between the two groups, but MAD decreased levels were significantly different. After 12 weeks of

intervention, TAC increased and MDA decreased in the pomegranate group when compared with the placebo group (Sohrab et al., 2015).

More recently, Boldaji et al. (2019) evaluated if consumption of pomegranate juice (PJ) improves oxidative biomarkers of haemodialysis patients. Forty-one haemodialysis patients were randomly assigned to one of the two groups: PJ-treated group receiving 100 mL of pomegranate juice immediately after their dialysis session three times a week and the control group receiving the usual care. Results showed an increase of TAC and a reduction of MAD levels in group treated with pomegranate juice. The protective effects of nutritional supplement in in haemodialysis patients were also previously investigated by Castilla et al. (2008). In this study, authors compared the effects of dietary supplementation with red grape juice (RGJ) and vitamin E on production of superoxide by circulating neutrophil NADPH oxidase. Thirty-two clinically stable patients were randomly distributed in 4 groups: RGJ, vitamin E, RGJ + vitamin E, and control. Participants in the RGJ group agreed to consume an oral supplement of 50 mL concentrated RGJ twice a day, at lunch and at dinner, for 2 weeks. Patients in the vitamin E group were instructed to take 800 IU vitamin E during each haemodialysis session for 2 weeks. The third group of patients (RGJ + vitamin E) received both 50 mL RGJ twice daily and 800 IU vitamin E as before, and the control group received neither supplement nor placebo during the study period. Results demonstrated that regular ingestion of concentrated red grape juice (RGJ) by haemodialysis patients reduces neutrophil NADPH-oxidase activity and plasma concentrations of oxidized LDL to a greater extent than does that of vitamin E.

The effects of antioxidant supplementation in diet was also analysed in healthy subject. In a randomized crossover study in healthy men (n= 27), Bub et al. (2003) determined the effects of 2 polyphenol-rich juices (330 mL per day) supplemented for 2 weeks on bioavailability of polyphenols, markers of antioxidative, immune status, and reduction of DNA damage. Juices contained a mixture of apple, mango and orange juice. In addition, juice A was rich in anthocyanin-providing aronia, blueberries, and boysenberries (76% w/w water), while juice B contained flavanol-rich green tea, apricot, and lime (78% w/w water). Juices provided 236 mg (A) and 226 mg (B) polyphenols with cyanidin glycosides (A) and epigallocatechin gallate (B) as major polyphenolic ingredients. Analysis showed no accumulation of plasma polyphenols after 2 weeks of juice supplementation. After juices consummation, the plasma MAD decreased with time during juice interventions and increased lymphocyte proliferative responsiveness. Additionally, a reduction of oxidative DNA damage in lymphocytes was observed.

Mathison et al. (2014) also suggested that consumption of polyphenol-rich foods is associated with lower risk from many chronic diseases and evaluated the effects of a single dose of cranberry beverage on oxidative stress. In a randomized, double-blind, placebo-controlled cross-over experimental design trial, 12 healthy participants consumed placebo, cranberry leaf extract beverage (CLEB), or low-calorie cranberry juice cocktail (LCJC). The results showed improvement of acute antioxidant status following cranberry and cranberry leaf beverage consumption and enhanced of

endogenous antioxidant enzyme systems. GSH and SOD activity were higher 24 h after consumption of LCJC compared to placebo. GPx activity was elevated 2 h after CLEB intervention, also suggesting increased endogenous antioxidant activity.

The effects of dietary intake of a blackcurrant juice on oxidative stress and vascular function was assessed by Khan et al. (2014). In a double-blind, placebo-controlled, parallel group study 66 healthy adults were randomly allocated to consume 250 mL blackcurrant juice drink four 6 weeks. Consumption of this drink high in vitamin C and polyphenols decrease oxidative stress and improve vascular health in individuals with habitually low dietary fruit and vegetable intake.

O'Byrne et al. (2002) compared the in *vivo* antioxidant efficacy of concord grape juice (CGJ) with that of α-tocopherol in healthy adults. 17 subjects were randomly assigned to receive either α-tocopherol/d (400 IU) and 15 subjects received CGJ (10 mL/kg/d) for 2 weeks. Serum oxygen radical absorbance capacity, plasma protein carbonyls, urinary F2-isoprostanes, and resistance of LDL to *ex vivo* oxidation were measured before and after supplementation as markers of antioxidant status and oxidative stress. The supplementation with CGJ increased serum antioxidant capacity and protected LDL against oxidation to an extent similar to that obtained with α-tocopherol supplementation but decreased native plasma protein oxidation significantly more than α-tocopherol. CGJ flavonoids are potent antioxidants that may protect against oxidative stress and reduce the risk of free radical damage and chronic diseases.

1.5 Diabetes Mellitus

Diabetes mellitus (DM) is a complex chronic disease characterized by metabolic disorders, such as hyperglycaemia, hyperinsulinemia, and hypertriglyceridemia associated with abnormalities in carbohydrate, fat, protein metabolism and results in chronic complications. The incidence of DM has greatly increased, and it is estimated that the numbers of patients have risen to more than 422 million until now, which will reach to 592 million in 2035 (Xu, 2018).

1.5.1 Glucose metabolism in physiological condition

Carbohydrates, molecules composed of carbon, hydrogen, and oxygen atoms, includes both simple (glucose and fructose) and complex sugars (starch, glycogen, and cellulose). The complex sugars are also called polysaccharides and are made of multiple monosaccharide molecules. Carbohydrates metabolism can be divided into: digestion, transport, storage, degradation, and biosynthesis (Stenesh, 1998).

Dietary carbohydrates of greatest importance are composed of hexoses such as sucrose (saccharose or table sugar), lactose (milk sugar), galactose (derived from fermented products) and maltose (derived from hydrolysis of starch) and also pentoses such as xylose and arabinose (from fruits) (Rosensweig & Herman, 1968).

Food digestion starts in the mouth through secretion of salivary α-amylase that hydrolyses α-1,4 linkage of starch and converts it to maltose. In the small intestine

pancreatic-amylase digests 60% of starches. Intestinal epithelial cell enzymes degrade 6-carbon (6C-) carbohydrates, in particular:

- sucrase degrade sucrose to glucose and fructose
- lactase degrade lactose to glucose and galactose
- maltase degrade maltose to two glucose molecules (Yeh et al., 1991).

Absorption of the 6C-carbohydrates from intestinal epithelium happens in two ways: passive and active transport systems. In the passive diffusion form, phosphorylation of carbohydrate (e.g., glucose or galactose) in the intestinal cells leads to their facilitated transfer to the circulation. Glucose-6-phosphate (G6P) and galactose-6- phosphate (Gal6P) are then dephosphorylated and enter the liver (Stevens et al., 1984). In the active diffusion form, carbohydrates utilizing a mobile carrier protein coupled with the sodium/potassium (Na+/K+) pump and against the gradient together with Na+ ion enter enterocytes. Therefore, the Na+/K+ pump using ATP as its source of energy exchanges 3 Na+ ions with 3 K+ ions (Stevens et al., 1984).

Glucose is used in different metabolic pathways for:

- ✓ stability of blood sugar in the hypoglycaemic state;
- ✓ energy supplier of the peripheral tissues;
- ✓ energy storage in the liver and skeletal muscle in the form of glycogen to be used in exercise;
- ✓ energy storage in the adipose tissue following conversion to triglycerides (Dashty, 2013).

The main metabolic pathways of glucose are (Stenesh, 1998):

1. *Glycogenesis*: process of glycogen synthesis with conversion of glucose into glycogen. Glycogen is a polymer of glucose residues that is linked by α-1,4 and α-1,6 glycosidic bonds. Therefore, it is the glucose storage molecule in the hepatocytes and skeletal muscle cells. Glycogen synthesis takes place in many tissues, but it is particularly important in liver and muscle where its magnitude and functional relevance is more significant. In humans, approximately 8% of the liver's weight is glycogen, especially after a diet rich in carbohydrates. This amount is considerably reduced after prolonged fasting. In skeletal muscle, glycogen contains approximately 1% of its weight.
2. *Glycogenolysis*: process of glycogen degradation and formation of glucose. Glycogenolysis happens in the liver and kidney to produce glucose for balancing the blood sugar; however, it produces G6P in muscle cells to be used as the energy supplier of myocytes. During the first 8–12 hours of fasting, glycogenolysis is the primary mechanism by which glucose is made available
3. *Glycolysis*: degradation of glucose into pyruvate and lactate. In the cells, glucose is processed through a sequence of reactions into smaller sugars, and the energy stored inside the molecule is released. In the glycolysis, was produces pyruvate, NADH, and ATP. Under anaerobic conditions, the pyruvate can be converted into lactate to keep glycolysis working. The chemical transformations during glycolysis include changes in the original substrate (glucose), with production of energy-rich metabolites for transfer of phosphoryl residues to ADP. This ability to generate ATP by substrate-level

phosphorylation mechanisms, without participation of oxygen and the respiratory chain, makes glycolysis a process of high physiological relevance.

4. *Pyruvate oxidative decarboxylation*: conversion of pyruvate (formed in glycolysis) into acetate.

5. *Krebs cycle*: oxidation of acetate to CO_2 and H_2O. Under aerobic conditions, pyruvate enters the Krebs cycle, also called the citric acid cycle or tricarboxylic acid cycle. In addition to ATP, the Krebs cycle produces high-energy $FADH_2$ and NADH molecules, which provide electrons to the oxidative phosphorylation process that generates more high-energy ATP molecules. For each molecule of glucose that is processed in glycolysis, a net of 36 ATPs can be created by aerobic respiration. Under anaerobic conditions, ATP production is limited to those generated by glycolysis. While four ATPs are produced by glycolysis, two are needed to begin glycolysis, so there is a net yield of two ATP molecules.

6. *Pentose phosphate or hexose monophosphate pathway*: alternative route for glucose oxidation.

7. *Gluconeogenesis*: glucose or glycogen production from no carbohydrate sources, using glucogenic amino acids, lactate, and glycerol as substrates. In conditions of low glucose, such as fasting, starvation, or low carbohydrate diets, glucose can be synthesized from lactate, pyruvate, glycerol, alanine, or glutamate. This process, called gluconeogenesis, synthesis of glucose from non-carbohydrate precursors. The major site of gluconeogenesis is liver.

Glucose homeostasis is tightly regulated by a number of hormonals, neural, and substrate glucoregulatory factors. The maintenance of the plasma-glucose level within a certain range under various physiologic conditions such as fasting, exercise, and feeding depends on the supply of sufficient amounts of substrates, on the actions of hormones regulating carbohydrate and intermediary metabolism as well as on the function of enzymes regulating the utilization and storage of glucose glycolysis, gluconeogenesis, and glycogen synthesis and breakdown (Dashty, 2013).

More specifically, the key glucoregulatory hormones include:

- ✓ Insulin, secreted from the β-cells of the pancreatic islets, exerts its action through binding to its receptors in insulin-sensitive tissues. This hormone lowers the plasma-glucose concentration by (a) suppressing hepatic as well as renal glucose production, (b) promoting glucose uptake by insulin-sensitive tissues, and (c) reducing circulating FFA level. The rate of insulin secretion from the β-cells of the pancreatic islets is itself regulated in response to circulating glucose concentration. Indeed, the insulin is secreted in increasing amounts as glucose concentration increases above 60 mg/dL (Aronoff SL, et al., 2006; Georga et al., 2018). In the post absorptive state, basal insulin secretion (5-10 μU/mL) regulates glucose primarily by inhibiting glucose and FFA release, while tissue glucose uptake is augmented during postprandial conditions (40-50 μU/mL) (Shrayyef & Gerich, 2010).

- ✓ Glucagon is secreted from the α-cells of the pancreatic islets in response to low plasma-glucose levels. Its glucose-raising action consists in enhancing hepatic glucose production by hepatic glycogenolysis. The subsequent glucose induced insulin secretion and insulin's direct effect on the liver suppresses the glycogenolytic response of glucagon (Georga et al., 2018).
- ✓ Epinephrine is secreted from the chromaffin cells of the adrenal medulla in response to a low plasma-glucose concentration. Its metabolic actions are, mostly, mediated by β2-adrenergic receptors. It increases hepatic glycogenolysis and, indirectly, hepatic as well as renal gluconeogenesis by increasing gluconeogenic precursors availability and plasma FFAs. Additionally, epinephrine reduces glucose uptake in skeletal muscle. The effect of epinephrine on glucose production, similarly to that of glucagon, is temporary; however, epinephrine is a potent hyperglycemic factor due to its sustained effect on glucose uptake (Georga et al., 2018).
- ✓ Growth hormone and cortisol are glucose counterregulatory hormones, which increase the synthesis of gluconeogenic enzymes and reduce glucose transport. Additionally, cortisol can impair insulin secretion. Unlike glucagon and epinephrine, their effect appears after several hours and they act synergistically to regulate plasma glucose concentration (Georga et al., 2018).
- ✓ Gastrointestinal inhibitory polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) incretin hormones which are effectively stimulated by meal ingestion. Both GIP and GLP-1 enhance insulin secretion, which explains the greater increase in plasma-insulin levels in response to an oral glucose load compared with an isoglycemic amount of intravenously infused glucose. GLP-1 potentiates insulin secretion according to plasma glucose concentration, and, also, suppresses postprandial glucagon secretion and gastric emptying, and promotes satiety reducing food intake and body weight (Georga et al., 2018). In addition, elevated plasma levels of FFAs stimulate hepatic and renal gluconeogenesis and inhibit glucose transport into skeletal muscle. The hormones that induce lipolysis, insulin, and plasma-glucose concentration constitute the major regulators of circulating FFAs (Georga et al., 2018).

For non-diabetic individuals in the fasting state, plasma glucose derived from glycogenolysis under the direction of glucagon, while the basal levels of insulin control glucose disposal. The role of insulin in suppressing gluconeogenesis and glycogenolysis is minimal due to low insulin secretion in the fasting state.

For patients with diabetes in the fasting state, plasma glucose derived from glycogenolysis and gluconeogenesis under the direction of glucagon. Exogenous insulin influences the rate of peripheral glucose disappearance and, caused of its deficiency in the portal circulation, not properly regulate the degree to which hepatic gluconeogenesis and glycogenolysis occurs (Aronoff et al., 2004).

For non-diabetic individuals in the fed state, plasma glucose is derived from ingestion of food. In the bi-hormonal model, glucagon secretion is suppressed through the action of endogenous insulin secretion, facilitated through the paracrine route. Furthermore, in

the fed state, insulin suppresses gluconeogenesis and glycogenolysis in the liver and promotes glucose disposal in the periphery. For patients with diabetes in the fed state, exogenous insulin is ineffective in suppressing glucagon secretion through the physiological paracrine route, with consequently increase of hepatic glucose production. A consequent, the glucose in the circulation exceeds the rate of glucose disappearance. The net effect is postprandial hyperglycaemia (Aronoff et al., 2004).

1.5.2 Prediabetic condition

The World Health Organization (WHO) has defined prediabetes as a state of intermediate hyperglycaemia using two specific parameters:

- ✓ impaired fasting glucose (IFG) defined as fasting plasma glucose (FPG) of 6.1-6.9 mmol/L (110 to 125 mg/dL);
- ✓ impaired glucose tolerance (IGT) defined as 2 h plasma glucose of 7.8-11.0 mmol/L (140-200 mg/dL) after ingestion of 75 g of oral glucose load or a combination of the two based on a 2 h oral glucose tolerance test (OGTT).

The American Diabetes Association (Nathan et al., 2009), on the other hand has the same cut-off value for IGT but has a lower cut-off value for IFG (100-125 mg/dL) and has additional haemoglobin A1c (HbA1c) based criteria of a level from 5.7 to 6.4% for the definition of prediabetes (preDM).

International Diabetes Federation declares that the prevalence of prediabetes is increasing worldwide and more than 470 million people will have prediabetes by 2030. It is reported that 5-10% of people per year with prediabetes will progress to diabetes, with the same proportion converting back to normoglycemia (Tabák et al., 2012).

In prediabetes condition was observed a simultaneous presence of insulin resistance and β-cell dysfunction, that start before glucose changes are detectable (Tabák et al., 2012). Weir & Bonner (2004) make a multistage model of diabetes development. In the first stage was observed a long period of insulin resistance accompanied by a compensatory increased rate of insulin secretion and increased β-cell mass. In the second stage, β cells are no longer fully compensating for increased insulin resistance. Consequently, fasting and post load glucose values are not completely maintained. This period probably starts when fasting and post-load glucose levels are still within the normal range and is usually accompanied by a decrease in acute insulin secretion at FPG concentrations of around 5·6 mmol/L (DeFronzo, 2009). The first and second stages therefore occur before the prediabetic phase. In the third stage of diabetes development, β-cells become unable to compensate for insulin resistance and consequently glucose concentrations start to increase rapidly. This period probably extends from prediabetes to manifest diabetes (Weir & Bonner 2004).

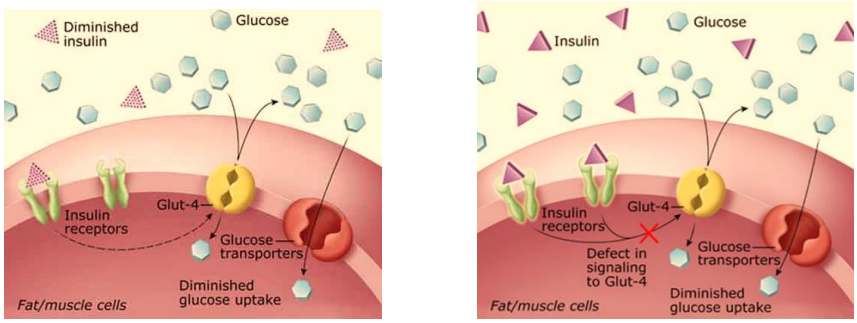
Fasting plasma glucose (FPG) values are determined by endogenous glucose production (EGP), and are used as markers of hepatic insulin resistance correlated with fasting glycaemia. During absorption of a glucose containing meal, the glucose concentrations changes, caused by intestinal absorption, suppression of EGP, and total body glucose uptake (De Fronzo, 2009). In people with normal glucose tolerance EGP is

greatly suppressed after glucose ingestion, contrary in prediabetes and diabetes subjects this suppression is less pronounced (Ferrannini et al., 2011).

In T2DM, total body glucose elimination decreased, correlated to muscle insulin resistance (Pendergrass et al., 2007). If insulin secretion was able to compensate for insulin resistance perfectly, no observable changes in glucose concentration would occur. This factor means that, by definition, β-cell dysfunction is already present in the prediabetic phase. However, β-cell function cannot be characterized only on the basis of insulin secretion without consideration of underlying insulin resistance. β cells respond to an increase in glucose concentration with a rise in insulin secretion that is dependent on whole body insulin sensitivity. The disposition index is a measure of insulin secretion after the underlying degree of insulin resistance (higher for healthy people and lower for prediabetic and diabetic individuals) has been accounted. Indeed, studies reported severely abnormal insulin secretion in prediabetic people, up to 80% decreases (Abdul-Ghani et al., 2006; Gastaldelli et al., 2004; Kahn 2003) and autopsies reported a 50% decrease in β-cell volume in subjects with IFG (Butler et al., 2003).

1.5.3 Classification of Diabetes Mellitus

DM is classified into Type 1 Diabetes Mellitus (T1DM); Type 2 Diabetes Mellitus (T2DM); Gestational Diabetes Mellitus (GDM); other specific type (Monogenic diabetes).



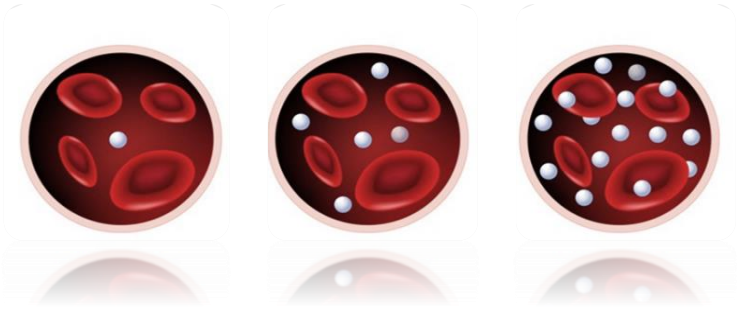
(a) (b)

Fig. 3 T1DM (a) and T2DM (b)

T1DM is an autoimmune disease of the pancreas and it is characterized by absolute insulin deficiency associated with pancreatic β-cells destruction. This diabetes is characterized by the presence of anti-glutamic acid decarboxylase (GAD), islet cell or insulin antibodies which identify the autoimmune processes leading to β-cell destruction (Baynest, 2015). In particular, in T1DM was observed autoimmune destruction of insulin producing cells in the pancreas by CD4+ and CD8+ T cells and macrophages infiltrating the islets. Furthermore, the involvement of monokines and TH1 cells that produce interleukins in the disease process has been reported. Frequently, occurs the onset of other organ-specific autoimmune diseases in affected individuals or their families. All diabetic patients will take insulin treatment to control their glycaemia. Both circulating insular cellular antibodies and detectable anti-insulin antibodies were detected in 85% of T1DM

patients before receiving insulin therapy. Most antibodies are directed against GAD within pancreatic B cells (Raju & Raju, 2010). All these phenomena induce autoimmune destruction of pancreatic β-cells, which in turn leads to a lack of insulin secretion with consequent metabolic disorders. Pancreatic α-cell function is also impaired with excessive glucagon secretion in T1DM patients. In a healthy subject, hyperglycaemia induces reduced glucagon secretion, but, in patients with T1DM, hyperglycaemia does not suppress this secretion. Indeed, high glucagon levels worsen metabolic defects due to insulin failure. Furthermore, insulin deficiency leads to uncontrolled lipolysis and high levels of free fatty acids (FFA) in the plasma, resulting in inhibition of glucose, lipid and protein metabolism (Baynest et al., 2015). The symptoms reported by the patient include polydipsia, polyphagia, polyuria, constipation, blurred vision, cramps, and candidiasis (Bearse et al., 2004).

Insulin resistance (IR) in muscle and liver, reduction in insulin secretion and β-cell failure represent the core pathophysiologic defects in development of T2DM, affecting nearly 95% of individuals. The progressive increase of these effects will contribute to change the state of normal to glycaemia, IFG, impaired glucose tolerance (IGT) and manifest diabetes (AlSaraj, 2015). Excessive hepatic glucose output is an important factor in the fasting hyperglycaemia of patients with T2DM (Figure 4).



(a) (b) (c)

Fig. 4 Bloond sugar level a) hypoglicemia b) normal level c) hyperglycemia

Gluconeogenesis represents the predominant mechanism responsible for increasing hepatic glucose output and it is correlated with fasting plasma glucose level. Insulin, regulates the metabolism of fats and carbohydrates in the body and allows glucose absorption from the circulation by fat tissue and skeletal muscles.

In patients with T2DM, the main causes are increased by gluconeogenesis. Indeed, the increased production of gluconeogenic precursors and increased hepatic FFA oxidation, can be responsible for gluconeogenesis (Durruty et al., 2019). In addition, in T2DM, it also occurs a reduction in suppression of hepatic glucose production after carbohydrate ingestion which plays a role in the damage in postprandial glucose homeostasis. In particular, fasting hyperglycaemia resulted from an increase in gluconeogenesis, while postprandial hyperglycaemia occurs due to reduced suppression of glycogenolysis and reduction in glucose uptake in skeletal muscle (AlSaraj, 2015). This mechanism will produce hyperglycaemic phenotype associated with insulin deficiency and IR. Thus, IR

will result in reduced insulin-mediated glucose uptake, incomplete inhibition of hepatic glucose production and impaired triglyceride uptake by fat. To exceed IR, β-cells will increase insulin secretion. In patients with T2DM, glucose production is accelerated and occurs in the presence of hyperinsulinemia. This shows that hepatic IR is the driving force of T2DM (Baynest, 2015). The main components of IR are alterations in glycaemic values, dyslipidaemia, hyperinsulinemia, hypertension and obesity. Glucose transporters (GLUT) are integral membrane proteins that mediate transport glucose (Figure 5). Alterations in glucose transport-4 (GLUT4) expression or regulation appear to contribute to the IR syndrome. Indeed, the increase of FFA, reduces glucose transport translocation by inhibition of PKCβ activity and lower GLUT phosphorylation (Durruty et al., 2019).

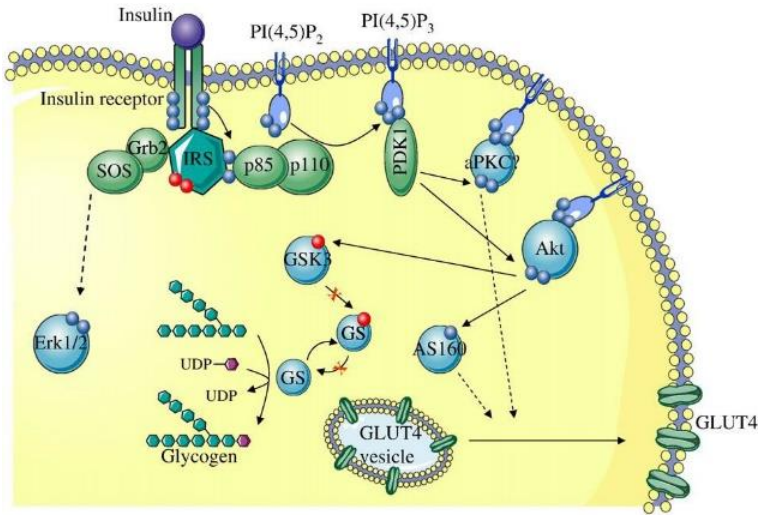


Fig. 5 Diagram illustrates the insulin regulated intracellular signal transduction cascade (Adapted from Frojdo et al., 2008)

1.5.4 Diabetes Mellitus complications

The chronic complications of diabetes mellitus determine multiple organs injury and most devastating consequence caused by long-term high level of blood glucose. The microvascular lesions can cause diabetic retinopathy, nephropathy and neuropathy while, the macrovascular complications include cardiovascular and cerebrovascular diseases (Asmat et al., 2016). There are micro- and macrovascular characterized by appearance of ulcer with distinctive symptom called diabetic foot. In general, complications of diabetes mellitus can be divided into:

- ✓ metabolic acute complications that include ketoacidosis, hypoglycaemia, and hyperosmolar non-ketonic coma.
- ✓ systemic late complications that include diabetic microangiopathy, nephropathy, diabetic neuro- and retinopathy and atherosclerosis.

In addition, oxidative stress has considered as a principal mediator in the progression of diabetic complications. Indeed, the improperly coupled electron transport within mitochondria results in formation of ROS in cells. During oxidative metabolism in the

mitochondria, a component of the oxygen used is reduced to water and the residual oxygen is transformed into an oxygen-free radical an important ROS, converted into $ONOO^-$, OH and H_2O_2 (Moussa, 2008).

Other mechanisms include activation of transcription factors, advanced glycated end products (AGEs), and protein kinase C (PKC). Hyperglycaemia can activate phospholipase D (PLD) and phospholipase C (PLC) that lead to formation of diacylglycerol (DAG), a physiological activator of PKC. After a hyperglycaemia peak, DAG keep in a high state fort 3 weeks. This dangerous metabolic route in diabetes, sustain the activation of PKC with consequent activation of nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase and ROS production (Volpe et al., 2018). Therefore, the increased ROS through these pathways leads to β-cell dysfunction and insulin resistance, responsible for cell damage and death (Pistocchi & Pop, 2015). Additionally, the high intracellular glucose in hyperglycaemia, that characterized T2DM, causes over activation of various molecular and biochemical pathways, contributing to increased ROS generation. Indeed, hyperglycaemia promote lipid peroxidation of LDL by a superoxide-dependent pathway, with consequent production of free radicals. Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to Amadori products and then advanced glycated end products (AGEs). These AGEs, through their receptors, inactivate enzymes altering their structures and functions, promote free radical production, quench and inhibit effects of NO. In particular, by increasing intracellular oxidative stress, AGEs activate the transcription factor NF-κβ and promote up-regulation of various NF-κβ controlled target genes (Maritim et al., 2003). Additionally, ROS are involved in the progression of insulin resistance ROS through disruption insulin-induced cellular redistribution of insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase (PI3-K). These processes alter insulin-induced GLUT4 translocation in 3T3-L1 adipocyte (Kaneto et al., 2010). High levels of FFA, inflammatory cytokines (eg, TNFα) and stress of the endoplasmic reticulum (ER) caused by hyperglycaemia and ROS, induced activation of the c-Jun N-terminal kinase (JNK) pathway. This activation lead insulin resistance and pancreatic β-cell dysfunction found in diabetes (Hotamisligil, 2005).

1.5.5 Therapeutic implication of pre-diabetes and Diabetes Mellitus

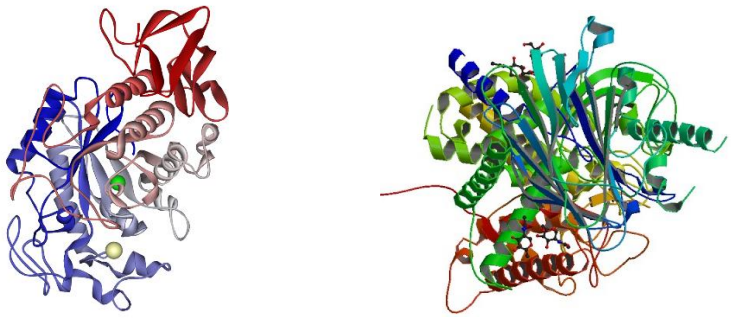
Prediabetes should be treated to prevent progression to diabetes, mitigate some of the potential results of progression to diabetes, and prevent the potential effects of prediabetes itself. Pharmacological intervention was based on antidiabetic drugs (Tabák et al., 2012).

Management of type 2 diabetes includes weight loss, healthy eating, regular exercise, diabetes medication or insulin therapy, and blood sugar monitoring.
The aim of antidiabetic therapy is to maintain blood sugar level closer to normal, reduce insulin resistance and thereby, delay or prevent diabetic complications.

Sulfonylureas are the first drugs used in 1954. Their mechanism of action includes the closure of ATP-sensitive K channels and increase in intracellular calcium. The effect reflects on the β-cells stimulating insulin secretion. In 1997, meglitinides were used. They

work like sulfonylureas, as prandial regulators of glycaemia, but they act more quickly and the duration of their effect in the body is shorter. In 1955, metformin was introduced in the United States. This drug, belonging to the biguanide family, acts as an insulin sensitizer associated or not with other hypoglycaemic drugs. In particular, metformin slows gluconeogenesis, reduces IR, increases glucose absorption and promotes its use by the gut. In year 2000 are introduced the thiazolidinediones, another group of insulin-sensitizing that reduce IR, especially in adipose tissue. They mechanism of action occurs through binding to the PPAR-Ϫ nuclear receptors. In 2005, dipeptidyl peptidase-4 (DPP-4) inhibitors are used help reduce blood sugar levels, but tend to have a very modest effect. They don't cause weight gain, but may cause joint pain and increase your risk of pancreatitis. More recently, sodium-glucose cotransporter-2 (SGLT2) inhibitors are introduced as hypoglycaemic agents. These drugs partially block renal glucose re-absorption and decrease glycaemia (Durruty et al., 2019).

One important therapeutic approach for suppressing postprandial hyperglycaemia is to reduce or down dietary carbohydrate digestion and absorption. The inhibition of the carbohydrates-hydrolysing enzymes, α-glucosidase and pancreatic α-amylase (Figure 6) in the digestive tract, determine a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise (Tundis et al., 2010). Acarbose represented the principal inhibitors of these enzymes, but its use is frequently associated with side effects such as diarrhoea, bloating, cramping, flatulence, and abdominal pain (Loizzo et al., 2018). Therefore, natural α-amylase and α-glucosidase inhibitors from the dietary plants can be used as an effective therapy for treating post-prandial hyperglycaemia with minimal side effects.



(a) **(b)**

Fig. 6 α-amylase (a) and α-glucosidase (b) enzymes
(Adapted from Ramasubbu et al., 1996; Barker MK & Rose DR, 2013)

α-Amylase (1,4-α-D-glucan-glucanohydrolase, EC 3.2.1.1) are a group of enzymes which catalyses the hydrolysis of α-1,4-glucan bonds in starch, and various other oligosaccharides. These enzymes are found in plants, animals, bacteria and fungi. In humans, the digestion of starch begins by the salivary amylase that degrade polymeric substrate into shorter oligomers. Subsequently, this digested material arrives in the gut where, by the pancreatic α-amylase, it is extensively hydrolysed into smaller

oligosaccharides and excreted in the lumen. These oligosaccharides reach the mucous layer of the brush border membrane, where intestinal α-glucosidases, hydrolyse it to glucose, which then enters the blood (Nair et al., 2013). Thus, this enzyme releases glucose from the non-reducing end of the substrate. Saqib (2008) studied the binding interactions between the competitive acarbose inhibitor and human α-glucosidase. The active site of human α-glucosidase is represented by a pocket formed mainly by the residues Asp^{398}, Asp^{587}, His^{645} and Arg^{571}. Furthermore, the residues Asp^{547}, Asp^{511}, Asp^{398}, Arg^{571} and His^{645} are important for a strong hydrogen binding interaction. By inhibiting α-amylase and α-glucosidase enzymes significantly delays postprandial hyperglycaemia, which may have beneficial effects on insulin resistance and glycaemic index control in diabetic patients. Thus, less glucose is absorbed because carbohydrates are not broken down into glucose molecules. Natural inhibitors of these enzymes from dietary plants can be used as an effective therapy for treating post-prandial hyperglycaemia with minimal side effects (Etxeberria et al., 2012).

Tadera et al. (2006) investigated the inhibitory activity of six groups of flavonoids on digestive enzymes and observed the relationship between the structures of the A, B and C rings and their inhibitory potential. This study established a relationship between the inhibitory activity and the increasing number of hydroxyl groups on the B ring (Flavonols: myrecetin > quercetin > kaempferol. Flavones: luteolin > apigenin).

The following structures enhanced the inhibitory activity: the unsaturated C ring, 3-OH, 4-CO, the linkage of the B ring at the 3 position, and the hydroxyl substitution on the B ring. Rat small intestinal α-glucosidase was weakly inhibited by many flavonoids, and slightly by the anthocyanidin and isoflavone groups. 3-OH and the hydroxyl substitution on the B ring increased the inhibitory activity. In porcine pancreatic alpha-amylase, luteolin, myricetin and quercetin were potent inhibitors with the IC_{50} values less than 500 μM. The 2,3-double bond, 5-OH, the linkage of the B ring at the 3 position, and the hydroxyl substitution on the B ring enhanced the inhibitory activity, while 3-OH reduced it. More recently, Liu et al. (2019) reported the application of eriocitrin for the prevention and treatment of diabetes by inhibiting α-glucosidase activity (Patent no. CN109806272A).

1.5.6 Nutritional supplements in clinical trials

Increasing evidence has demonstrated that the combinations of several unhealthy lifestyle factors, including a sedentary lifestyle, unhealthy diets, overweight/obesity, and smoking, were responsible for 90% of T2DM cases (Chen et al., 2011). For this reason, strategies focused on lifestyle modification and the promotion of a healthy diet to prevent T2DM have been identified as a cornerstone (Guasch-Ferré et al., 2017), with relative-risk reduction of 40-70% (Tabák et al., 2012). Nutraceutical products from plants have been reported to reduce hyperglycemia and lipid disorders in individuals with T2DM or with a predisposition to T2DM (Kaleem & Ahmad, 2018).

A nutraceutical product, Diabetinol®, rich in *Citrus* flavanones (nobiletin, 49% and tangeritin, 13%), limonoids, tocotrienols and vitamin E significantly reduced peak

hyperglycemic response, TC, and LDL-C levels and decreased HbA1c levels after 3 months supplementation in subjects with mild impaired fasting glucose (Judy et al. 2010).

Similar results were observed in subjects with greater severity of impaired fasting glucose (≤15.4 mmol/L) in a 24-week, randomized, double-blind, placebo-controlled, parallel study carried out by Evans (Evans et al. 2015).

The effect of standard bergamot polyphenolic fraction (BPF®) as well as of its phytosomal formulation (BPF Phyto), in patients with type 2 DM and hyperlipemia was investigated by Mollace et al. (2019). This randomized, double blind, placebo-controlled study was carried out in 60 patients suffering from T2DM and mixed hyperlipemia. In the groups receiving BPF® and BPF Phyto, was observed a significant reduction of fasting plasma glucose, serum LDL cholesterol and triglycerides accompanied by increased HDL cholesterol. However, when comparing the pharmacokinetic profile of naringin (the major component of BPF®) and its metabolites, in patients treated with BPF Phyto, an at least 2,5-fold increase in its absorption was found, confirming in human studies the better profile of BPF Phyto compared to standard BPF®.

Previously, Di Folco et al. (2018) reported the effects of a bergamot fruit phytocomplex registered with Patent no. EP3116520A1 as dietary supplement in subjects with the metabolic syndrome. Each tablet, provided by ESSERRE PHARMA, includes: 200 mg bergamot juice dry extract, 120 mg phytosterols, 80 mg artichoke leaf extract and 20 mg vitamin C. After 6 months, patients in the intervention group showed a significant reduction in fasting blood glucose, compared to the simple dietary intervention alone.

A significant reduction in 2-h post-glucose insulin level and improvement of insulin resistance were observed in patients with metabolic syndrome compared to placebo following 12 weeks administration with 237 mL of grapefruit juice three times a day before each meal (Fujioka et al., 2006).

In randomized and double-blind pilot clinical trial, Campbell-Tofte et al. (2011) investigated the anti-diabetic efficacy of the *Rauvolfia-Citrus* (RC) tea in type 2 diabetes patient. The RC tea was prepared by boiling *Rauvolfia vomitoria* foliage and *Citrus aurantium* fruits. At the end of the 4-month treatment period, the treated group with RC showed a decrease in 2-h postprandial plasma glucose, the improvement in blood glucose clearance with reduction in HbA_{1c} and in fasting plasma glucose.

With carbohydrate-hydrolysing enzyme inhibitory activity, act the extract of *Castanea sativa* that administered at doses of 300 and 600 mg with 200 g of boiled rice after an overnight fasting in 11 healthy volunteers showed a dose-dependent reduction in plasma glucose levels (11 and 23%, respectively). Moreover, this extract did not produce adverse effects at doses of 2000 mg/kg body weight/day (Tsujita et al., 2008).

In a randomized, double-blind placebo-controlled trial supplementation with 5 g/day of mulberry leaf aqueous extract (MLAE) for 4 weeks in 36 subjects with impaired fasting glucose (IFG) tolerance was assessed. Results showed that supplementation improved postprandial glycaemic control in individuals with IFG tolerance. Additionally, the incremental area under the curve for insulin was significantly lower in the MLAE group than in the placebo group (Kim et al., 2014). Successively, Thaipitakwong et al. (2019)

reported that supplementation with 12 mg of mulberry leaves three times daily for 12-week attenuated postprandial hyperglycaemia by decreased fasting plasma glucose, glycated hemoglobin, and ameliorated insulin resistance without serious side effects.

Huseini et al. (2011) conducted a randomized double-blind placebo-controlled clinical trial with hyperlipidaemic (hypercholesterolemic and/or hypertriglyceridemic) T2DM patients aged 40 to 60 years. A group of thirty patients was treated with *Aloe* gel (treated to remove aloin and anthraquinones, which have laxative effects) capsules at the dose of 300 mg every 12 hours by the oral route for 2 months the other group with placebo. The results suggest that *Aloe* gel may be a safe anti-hyperglycaemic and anti-hypercholesterolemic agent for hyperlipidaemic T2DM patients. Indeed, the *Aloe* gel lowered the fasting blood glucose, HbA1c, total cholesterol, and LDL levels without adverse effects. Subsequently, the effects of *Aloe vera* supplementation in subjects with prediabetes/metabolic syndrome were confirmed by Devaraj et al. (2013). In this double-blind, placebo-controlled study two aloe products UP780 (standardized with 2% aloesin, 500 mg capsules,) and AC952 (500 mg capsules), in patients with prediabetes were tested. After 8-week It was reported that both AC952 and UP780 could markedly reduce fasting glucose and improve glucose tolerance and lipoprotein levels in the plasma. However, the reduction of oxidative stress marker urinary F2-isoprostanes was solely noted for UP780 compared with the placebo.

A phytocomplex derived from maqui berries commercialized as Delphinol® was administered at dose of 180 mg/day, in 31 subjects with pre-diabetes. This phytocomplex significantly lowers blood glucose and improves blood lipid profile in prediabetic individuals in three-month clinical trial, in absence of side effects (Alvarado et al., 2016). Previously investigations, suggested that Delphinol® intake (200 mg/day) prior to rice consumption significantly lowered post prandial blood glucose and insulin increase in ten volunteers with moderate glucose intolerance (Hidalgo et al., 2014).

Curcumin is proposed to be used as one of the interventions in pre-diabetes therapy to prevent the progression of T2DM due to its proven benefits and safety profile (Chuengsamarn et al., 2012). In this randomized, double blinded, placebo-controlled human clinical trial study, no subjects treated with curcumin (six capsules per day with curcuminoid content of 250 mg) for a period of 9 months developed T2DM; whereas 16.4% of subjects from placebo group were found to develop T2DM. Additionally, the subjects treated with curcumin showed better overall beta-cells function with lower values of C-peptide and higher values of HOMA-b compared to placebo subjects.

Mahmoud et al. (2016) evaluated the effects of black tea ingestion on the secretion of inflammatory cytokines and metabolic biomarkers in 30 patients with T2DM. The results demonstrated that treatment with black tea at 200 or 600 mL per day, for 12 weeks, determined reduction in glycosylated haemoglobin levels, total serum cholesterol levels and the markers of oxidative stress. The effect of the chamomile tea on glycaemic control and antioxidant status in patients with T2DM was analysed by Zemestani et al. (2016) in a single-blind randomized controlled clinical trial. Chamomile tea, at 3 g/150 ml, 3 times per day, for 8 weeks decreased the concentration of serum glycosylated hemoglobin,

MDA, insulin, and improved insulin resistance in 64 patients with T2DM. Additionally, intake of chamomile tea increased total antioxidant capacity of SOD, GSH, and CAT by 6.81, 26.16, 36.71, and 45.06%, respectively.

The effect of cinnamon supplementation was investigated by Tangvarasittichai et al. (2015) that performed a randomized, double blind, placebo-controlled trial in 49 patients with T2DM. After intake of 500 mg, capsules for 60 days, marked reduction in insulin resistance, oxidative stress and inflammation was observed while insulin sensitivity and TAC were markedly increased.

1.6 Obesity

Obesity is defined as chronic metabolic disorder characterized by accumulation of abnormal or excessive fat that alters health and increases mortality. Humans obtain all energy from ingested food, store it as high-energy molecules, and expend it during basal metabolic functions, activity, and thermogenesis. In the steady state, the body's energy inputs balance the sum of energy outputs. However, when energy consumption exceeds energy expenditure, about 60-80% of excess energy, is converted into TGs (Oussaada et al., 2019). Currently, 1.3 billion people around the world are overweight or obese. As such, obesity is the fifth greatest cause of non-communicable diseases.

1.6.1 Physiology of fat metabolism

Fats or triglycerides are ingested as food or synthesized by adipocytes or hepatocytes from carbohydrate precursors. Lipid metabolism entails the oxidation of fatty acids to either generate energy or synthesize new lipids from smaller constituent molecules. Lipid metabolism is associated with carbohydrate metabolism, as products of glucose can be converted into lipids (Watts & Ristow, 2017).

Lipid metabolism begins in the intestine where ingested triglycerides are broken down into smaller chain fatty acids and into monoglyceride molecules (Hall, 2015).

When food reaches the small intestine in the form of chyme, a digestive hormone called cholecystokinin (CCK) is released by intestinal cells in the intestinal mucosa. CCK stimulates the release of pancreatic lipase from the pancreas and stimulates the contraction of the gallbladder to release stored bile salts into the intestine. CCK also travels to the brain, where it can act as a hunger suppressant (Johnson, 2013). Together, the pancreatic lipase and bile salts break down triglycerides into free fatty acids. These fatty acids can be transported across the intestinal membrane (Hofmann, 1963). However, once they cross the membrane, they are recombined to again form triglyceride molecules. Within the intestinal cells, these triglycerides are packaged along with cholesterol molecules in phospholipid vesicles called chylomicrons. The chylomicrons enable fats and cholesterol to move within the aqueous environment of your lymphatic and circulatory systems. Chylomicrons leave the enterocytes by exocytosis and enter the lymphatic system via lacteals in the villi of the intestine. From the lymphatic system, the chylomicrons are transported to the circulatory system. Once in the circulation, they can

either go to the liver or be stored in fat cells (adipocytes) that comprise adipose (fat) tissue found throughout the body (Smit et al., 2013).

To obtain energy from fat, triglycerides must first be broken down by hydrolysis into their two principal components, fatty acids and glycerol. This process, called lipolysis, takes place in the cytoplasm (Stryer, 1995). The resulting fatty acids are oxidized by β-oxidation into acetyl-CoA (acetyl coenzyme A) CoA, which is used by the Krebs cycle (Houten & Wanders, 2010). The glycerol that is released from triglycerides after lipolysis directly enters the glycolysis pathway. Since one triglyceride molecule yields three fatty acid molecules with as much as 16 or more carbons in each one, fat molecules yield more energy than carbohydrates and are an important source of energy for the human body (Park & Chalfant, 2014). Triglycerides yield more than twice the energy per unit mass when compared to carbohydrates and proteins. Therefore, when glucose levels are low, triglycerides can be converted into acetyl CoA molecules and used to generate ATP through aerobic respiration (Park & Chalfant, 2014).

The breakdown of fatty acids, β-oxidation, begins in the cytoplasm, where fatty acids are converted into fatty acyl CoA molecules. This fatty acyl CoA reacts with carnitine to form a fatty acyl carnitine molecule, which helps to transport the fatty acid across the mitochondrial membrane (Park & Chalfant, 2014). Once inside the mitochondrial matrix, the fatty acyl carnitine molecule is converted back into fatty acyl CoA and then into acetyl CoA. The newly formed acetyl CoA enters the Krebs cycle and is used to produce ATP in the same way as acetyl CoA derived from pyruvate (Houten & Wanders, 2010).

When glucose levels are plentiful, the excess acetyl CoA generated by glycolysis can be converted into fatty acids, triglycerides, cholesterol, steroids, and bile salts. This process, called lipogenesis, creates lipids from the acetyl CoA and takes place in the cytoplasm of adipocytes and hepatocytes (Kersten, 2001). When you eat more glucose or carbohydrates than your body needs, your system uses acetyl CoA to turn the excess into fat. Although there are several metabolic sources of acetyl CoA, it is most commonly derived from glycolysis. Acetyl CoA availability is significant, because it initiates lipogenesis. Lipogenesis begins with acetyl CoA and advances by the subsequent addition of two carbon atoms from another acetyl CoA; this process is repeated until fatty acids are the appropriate length. Since this is a bond-creating anabolic process, ATP is consumed. However, the creation of triglycerides and lipids is an efficient way of storing the energy available in carbohydrates. Triglycerides and lipids, high-energy molecules, are stored in adipose tissue until they are needed (Ahmadian et al., 2007).

Although lipogenesis occurs in the cytoplasm, the necessary acetyl CoA is created in the mitochondria and cannot be transported across the mitochondrial membrane. To solve this problem, pyruvate is converted into both oxaloacetate and acetyl CoA. Two different enzymes are required for these conversions. Oxaloacetate forms via the action of pyruvate carboxylase, whereas the action of pyruvate dehydrogenase creates acetyl CoA. Oxaloacetate and acetyl CoA combine to form citrate, which can cross the mitochondrial membrane and enter the cytoplasm. In the cytoplasm, citrate is converted back into oxaloacetate and acetyl CoA. Oxaloacetate is converted into malate and then into

pyruvate. Pyruvate crosses back across the mitochondrial membrane to wait for the next cycle of lipogenesis. The acetyl CoA is converted into malonyl CoA that is used to synthesize fatty acids (Tsiloulis & Matthew, 2015).

1.6.2 Classification of obesity

The most common classification is based on collection and analysis of body mass index (BMI, kg/m2) intervals related to mortality risk (Olszewska et al., 2018):

- ✓ class I for a BMI of 30-34.9 associated with a moderate risk;
- ✓ class II for a BMI of 35-39.9 with a high risk;
- ✓ class III for a BMI of 40 or higher associated with a very high risk of mortality.

In addition, body fat can be deposited at a visceral and abdominal level (a serious risk of metabolic disorders), characterizing central adiposity, or subcutaneously, characterizing peripheral adiposity.

In etiological terms, obesity is classified as:

- ✓ primary: it follows a positive energy balance. The identification of the primary causes of this imbalance remains difficult and includes most of the cases usually diagnosed after the causes of secondary obesity have been excluded.
- ✓ secondary: also called iatrogenic and it is secondary to pharmacological treatments (antipsychotics, antiepileptic, antidepressants, steroids, etc.) or to some diseases (hypothyroidism, Cushing's syndrome, hypothalamic defects, etc).

1.6.3 Nutritional feedback

Adipose tissue, in addition to TG preservation, represents an important metabolic and endocrine organ that secretes cytokines, adipocytokines, growth factors and hormones involved in energy homeostasis and systemic insulin sensitivity. Indeed, the pathogenesis of obesity is complicated and multifactorial, implying the dysfunction of different peptides, hormones, and neuronal signals from the brain, stomach, pancreas and adipose tissue. These signals regulate food intake and energy expenditure and maintain energy homeostasis in our bodies (Mohamed et al., 2014).

Leptin and ghrelin are internal mediators that play an important role in the regulation of body weight and energy balance through inhibition food intake and stimulating energy expenditure. Ghrelin is a gastrointestinal hormone and is produced by the stomach modulating short-term appetitive control; its levels increase during fasting and decrease postprandial, in response to nutrient intake. This mechanism controlled by the sympathetic nervous system. Fasting ghrelin levels and BMI are negatively correlated. In fact, human obesity is associated with reduced postprandial ghrelin suppression (Oussaada et al., 2019). Leptin is predominantly produced by white adipose tissue to signal fat storage reserves in the body and mediates long-term appetitive controls (Mohamed et al., 2014). These mediators, activate neurons that synthesize anorexigenic peptides, such as pro-opiomelanocortin (POMC), suppress the activity of orexigenic neurons and counterbalances the effects of ghrelin. Obesity is characterized by leptin resistance, caused from hypothalamic inflammation, gliosis and mutations in genes

encoding components of the leptin melanocortin pathway (Oussaada et al., 2019). The role of insulin is significant in the context of nutritional feedback. In fact, its effects on the hypothalamus promote satiety after increased release of dopamine from the striatum.

Obesity is characterized by insulin resistance, a condition that can also develop in the brain, suggesting that insulin-mediated nutritional feedback could be compromised by obesity (Kim & Feldam, 2012).

1.6.4 Treatment of obesity

Possible mechanisms of weight loss include:

- ✓ Appetite control. Serotonin, histamine and dopamine through interaction with their own receptors, control hunger and satiety. Sibutramine is an anti-obesity drug that works as an appetite suppressant. However, its use is limited by its side effects such as dry mouth, constipation and insomnia (Tziomalos et al., 2009).
- ✓ Induction of non-shivering thermogenesis by simulation of energy expenditure. Thermogenesis is regulated by the release of protons generated in oxidative phosphorylation, bypassing the generation of ATP and activating mitochondrial uncoupling protein-1 (UCP-1) which dissipates energy in the form of heat. Additionally, also UCP-3 mediates the thermogenesis regulated by the leptin, thyroid hormone, and β-adrenergic receptor agonist (Rapusinghe et al., 2016). Previously Gong et al. (2000) demonstrated the involvement of the UCP family in thermogenesis. In fact, mice that excessively express UCP family were resistant to diet-induced obesity but were sensitive to cold due to lack of thermogenesis.
- ✓ Inhibition of adipocyte differentiation. In a diabetic subject, there is an increase in size and differentiation of adipocytes caused by fat accumulation. Compounds, able to block this process and induce apoptosis in mature adipocytes are considered potent anti-obesity agents (Yun, 2010).
- ✓ Stimulation of triacylglycerol hydrolysis and subsequent release of fatty acids. Lipolysis reduces the accumulated fat but it is necessary to intervene on the β-adrenergic receptor agonist to oxidize the released fatty acids (Langin, 2006).
- ✓ Use of peroxisome proliferator gamma receptor agonists (PPAR-γ). This receptor stimulates adipose differentiation and therefore, these agonists can improve insulin resistance, adiposity and dyslipidaemia (Rapusinghe et al., 2016).
- ✓ Reduction of fat absorption. In the gastrointestinal tract, before absorption, fats are subjected to the action of pancreatic lipase that performs essential roles in digestion, processing of dietary lipids to monoglycerides and FFA in humans. Lipoprotein lipase is a digestive subclass of the esterases that catalyses the hydrolysis of core TGs in chylomicrons and very low-density lipoproteins (VLDL), producing chylomicron remnants and intermediate-density lipoproteins (IDLs), respectively. Thus, lipase decreased digestion and absorption of ingested fats lead to overall decreased caloric absorption ultimately leading to decreased obesity (Balaji et al., 2016). The inhibitor of lipase covalently binds at the active site to the hydroxyl group and forms many stable complexes (Figure 7). Therefore,

this confirms the structural modifications on the enzyme and exposes catalytic site. Once active site is visible, the serine residue containing hydroxyl groups are acylated and thus causing irreversible inactivation of the enzyme. The inactivated enzyme does not play any role on hydrolysing fats into fatty acids and monoglycerides and thus the undigested TG's are excreted through faeces (Rajan et al., 2020). The pancreatic lipase inhibitors are clinically approved for obesity treatment such as, Orlistat. This drug act by through a covalent bond at the lipase's active site (serine). Although, Orlistat has some unpleasant gastrointestinal side effects like oily spot-ting, faecal urgency or incontinence, flatulence, liquid stools and abdominal cramping.

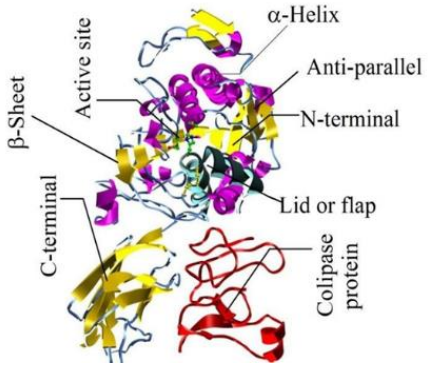


Fig. 7 Pancreatic lipase (Adapted from Mukherjee, 2014)

Obesity is often associated with oxidative stress, obesity and T2DM (Figure 8) (Xu et al., 2018). With IR development, the insulin activity, as an antilipolytic hormone, decreases. Consequently, the hyperinsulinemia activates the lipoprotein lipase (LP) and caused FFA released from lipoprotein triglycerides hydrolysis.

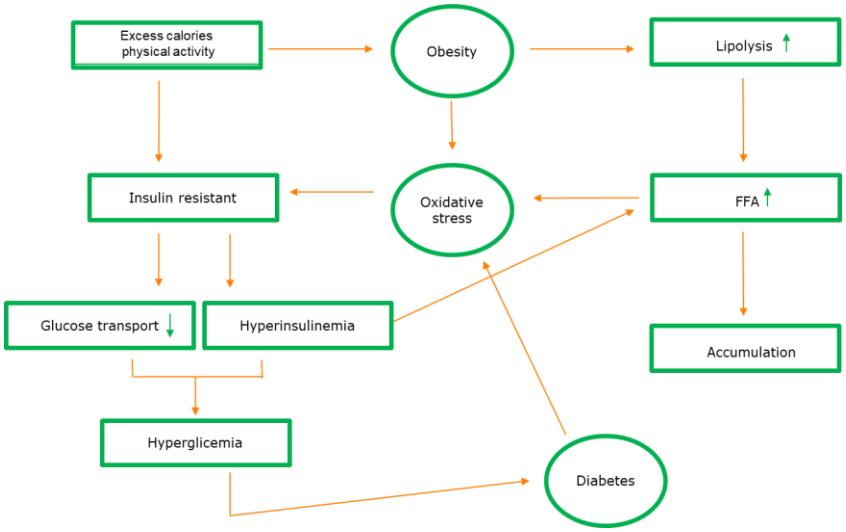


Fig. 8 Relation oxidative stress, diabetes and obesity

Liver maintain stable level of blood glucose through the balance between glycogenesis and glycolysis of stored glycogen. The fatty deposit in liver is accompanied by the build-up of IR. Indeed, high level of FFA determine the accumulation of diacylglycerol that inhibit insulin action by protein kinase C activation. This mechanism, interferes with insulin signal transduction through serine phosphorylation of insulin receptor (Xu, 2018).

1.6.5 Nutritional supplements in clinical trials

Nutritional supplements have been claimed to increase energy metabolism, reduce fat absorption, and increase fat oxidation all of which thereby increase weight loss (Jeukendrup & Randell, 2011). The majority of the ingredients used in these nutritional supplements are from plant origin and commonly referred as phytochemicals. Their use can be an excellent alternative for anti-obesity drugs. A variety of natural products, including crude extracts and isolated pure natural compounds can prevent diet-induced obesity reducing body weight. Research on natural products has recently regained importance with the growing understanding of their biological significance. Botanical food supplements marketed for weight and fat loss in obese subjects will be one of the most important items in marketed nutraceuticals (Marrelli et al., 2020).

Bitter orange *(C. aurantium)* is recognized as a supplement thermogenic agent. The active ingredient of bitter orange is the alkaloid synephrine, that increase metabolism and induce lipolysis (Nuffer & Nuffer, 2018). The metabolic effects of Xenadrine EFX® was demonstrated by Kalman et al. (2002) in a double-blind cross-over study involving 6 healthy human subjects who received two capsules of 12 mg *p*-synephrine. A significant increase in resting metabolic rate was observed 1 hour after ingestion of the product relative to the placebo control, and no subjective complaints or adverse events were reported. The weight loss effects of Xenadrine™, a product that contains 20 mg of ephedrine, 5 mg of *p*-synephrine, 200 mg of caffeine and 15 mg of salicin, was investigated in 30 healthy overweight subjects in a placebo-controlled double-blinded protocol. Patients were treated twice daily for 8 weeks. Results evidenced a great loss of weight than the control group without any significant changes in systolic or diastolic blood pressure, heart rate, etc (Stohs et al., 2012). Additionally, the same authors reported a study on a system (Ultra Slim Down®) that consisted of two products. One product contained 125 mg hydroxycitric acid (Citrimax^{TM}), 125 mg bitter orange extract (Advantra Z®) and 50 mg kola nut extract. The second product contained 344 mg chitosan. Thirty-two overweight subjects were treated with one capsule of each products in conjunction with each meal, diet and exercise program for 10 weeks. The consumption of the products in combination with diet and exercise was most effective with weight loss of 6.59 kg. Previously, 12 human subjects were treated with bitter orange extract for 28 days (Gurley et al. 2004). The daily consumption of *p*-synephrine was 30.6 mg. The supplement containing *C. aurantium* extract did not cause significantly inhibition of cytochrome P450 enzyme activities in human subjects, and consequently minimal risk herb-drug interactions. Additionally, the bitter orange extract had no significant effect on

CYP1A2, or CYP3A4, the major drug-metabolizing cytochrome enzymes and no adverse effects were observed.

Like bitter orange also caffeine is considered as a thermogenic and it is investigated for potential use in body weight reduction by inhibition of the phosphodiesterase-induced degradation of intracellular cyclic AMP. More recently, Clark et al. (2020) suggested that thermogenic fitness drink formulas containing 100 and 140 mg of caffeine was effective in increasing resting energy expenditure (REE) and that a 40 mg of caffeine difference between the tested formulas may impact REE and resting fat oxidation in healthy individuals (thirty-two) within 60 min of ingestion. Davoodi et al. (2014) evaluated the effect of caffeine treatment (5 mg/Kg/day) with calorie shifting diet (CSD) on weight loss of 60 female subjects. The results indicated that combination of caffeine treatment with CSD could be an effective alternative approach to weight and fat loss with improved tolerance of subjects to the new diet.

Liu et al. (2013) evaluated the effects of combination caffeine/ephedrine and leptin A-200 on visceral fat mass and weight loss over 24 weeks. In this study, 90 obese subjects received 200 mg caffeine/20 mg ephedrine. This treatment was a modestly effective weight loss agent and produces significant reductions in fat mass.

The importance of *Citrus* supplementation in obesity management was also investigated by Dallas et al. (2013). The authors demonstrated the efficacy and safety effects of Sinetrol-XPur (polyphenolic *Citrus* dry extract) in weight management, metabolic parameters, and inflammatory, glycemic and oxidative status. Sinetrol-XPur is a polyphenolic rich fruit extract (red orange, grapefruit, sweet orange, and guarana). It was standardized to contain at least 90% of total polyphenols (expressed as catechin), at least 20% of total flavanones (expressed as naringin) and between 1% and 3% of natural caffeine. The dry extract was packaged in red gelatine capsules (450 mg per capsule). Identical-looking capsules were filled with 450 mg of maltodextrin and used as placebo. In a 12-week, randomized, double-blind, placebo-controlled trial, Sinetrol-XPur was given to overweight subjects twice daily with meals in the tested group (n = 47) *versus* a placebo group (n = 48). Subjects were instructed to take one capsule at breakfast and one capsule at lunch for a total of two capsules per day or 900 mg. Waist and hip circumference and abdominal fat were decreased in the Sinetrol-XPur group as compared with the placebo group. Inflammatory markers (C-reactive protein) were reduced. Oxidative stress was lowered as seen by the reduction of and the increase in superoxide dismutase and glutathione. No adverse effects were observed.

More recently, Kamel et al. (2019) reported the results on double-blind, placebo-controlled study in which *C. reticulata* peel water extract was administered to obese adolescents. This extract is characterized by hesperidin, naringin, acacetin, rutin and quercetin as main flavonoids. Group A (n= 40) received 800 mg of dry extract daily whereas group B (n= 40) received placebo and both groups received three meals (2000 kcal/day) throughout the study. Results evidenced a significant reduction in BMI and waist circumference after 4 and 8 weeks of supplementation when compared with placebo group. Additionally, a reduction of TC and TG levels was observed. Moreover, the basal

metabolic rate was not significantly increased which suggested the *Citrus reticulate* peel extract was to be well-tolerated and effective ingredient for weight management.

C. bergamia is an endemic plant growing in a limited part of the Ionian coast of Calabria (Italy). It was characterized by a unique content of flavonoids and glycosides, such as neoeriocitrin, neohesperidin, naringenin, rutin, neodesmine, and poncirin. Di Folco et al. (2018) investigated the effects of a bergamot fruit phytocomplex (200 mg bergamot juice dry extract, 120 mg phytosterols, 80 mg artichoke leaf extract and 20 mg vitamin C) registered with Patent no. EP3116520A1 as dietary supplement for the management of obesity. After 6 months patients in the intervention group showed a significant reduction of total cholesterol, LDL-cholesterol, triglycerides, blood glucose, C reactive protein (CRP) and a significant increase in the HDL-Cholesterol levels. Supplementation of a mixture of *Imperata cylindrical*, *Citrus unshiu markovich*-Hesperidin, and *Evodia officinalis Dode*-Evodiamine for 12 weeks significantly reduced the body weight, body fat mass and waist circumference in overweight subject (Cho et al., 2017).

Previously, Silver et al. (2011) reported that eighty-five obese adults (BMI 30-39.9) were randomly assigned to (127 g) grapefruit, grapefruit juice or water preload for 12 weeks after completing a 2-week caloric restriction phase. Subjects showed 7.1% weight loss overall, with significant decreases in percentage body, trunk, android and gynoid fat, as well as waist circumferences (-4.5 cm). The effects of grapefruit on body weight and metabolic syndrome in 91 obese patients were also investigated by Fujioka et al. (2006). This randomized, double-blinded, placebo-controlled study comparing fresh grapefruit, grapefruit juice, grapefruit capsules, and/or placebo capsules in obese subjects. The supplementation results after 12 weeks determined decrease of body weight: -1.6 kg in the fresh grapefruit group, -1.5 kg in the grapefruit juice group and -1.1 kg by the grapefruit capsule compared to placebo -0.3 kg. Additionally, the insulin resistance was improved in fresh grapefruit group.

A literature data on nutritional supplements for the treatment of obesity evidence the efficacy of other products. More recently, Hou et al. (2020) reported that consumption of high-fat diet supplemented with cooked mung bean (30%, w/w) for 12 weeks effectively alleviated body weight gain and lipid metabolic disorders, which was accompanied by a decrease in hepatic steatosis and adipocyte size. Previously, Thom et al. (2000), conducted a randomized, double blind, placebo-controlled trial was in 40 overweight and obese subjects that received Phase II, an aqueous extract of white kidney bean, for 12-weeks. The group was treated with two tablets of the product after the three main meals, each one containing 200 mg Phase II, 200 mg inulin and 50 mg *Garcinia cambogia* Gaertn. extract. At the end of the study a significant reduction in weight and fat mass were demonstrated with similar tolerability in both treat and placebo groups.

The effects of green coffee bean extract (GCBE) consumption on serum oxidized LDL-cholesterol and total antioxidant capacity on patients with dyslipidaemia were evaluated by Salamat et al. (2018). In this randomized, placebo-controlled, clinical trial, 70 male subjet (age range 30–55 years) were treated with 800 mg/day GCBE supplements

or placebo for 8 weeks. Compared with the placebo, the extract intake led to a significant LDL-cholesterol and a significant increase in total antioxidant capacity concentration. Similarly, in randomized triple -blind, placebo controlled parallel trial Zare et al. (2018) investigated the effects of supplementation with cinnamon (1g /day for 13 week) in diabetic patients. The supplement led to improvement of all anthropometric (BMI, body fat, and visceral fat), glycaemic (FPG, 2hpp, HbA1C, Fasting Insulin, and Insulin Resistance), and lipids (Cholesterol Total, LDL-c and HDL-c) outcomes in the cinnamon group compared to the placebo group. All observed changes were significantly more prominent in patients with higher BMI (BMI≥27).

Daily administration of Xanthigen (300 mg pomegranate seed oil + 300 mg brown seaweed extract containing 2.4 mg fucoxanthin) for day resulted in a significant weight loss compared with placebo after 16 weeks. The authors also reported increases in resting energy expenditure, decreases in body and liver fat content and improvements in the plasma lipid profile. Weight reductions were about 5 kg more in the supplemented group compared with the placebo (Abidov et al., 2010).

Psyllium seed husk, is one of the most widely used fiber supplements in the world. Psyllium is better tolerated than other fiber supplements because it causes less abdominal bloating; thus, it has advantages over other types of soluble fiber (Blackwood et al., 2000). Recently, Pal et al., (2019) reported that Psyllium represented an effective adjunct to dietary intervention in the control of body weight, body composition, cholesterol, glucose, insulin, and triglyceride levels both in animal and human studies. In particular, in human studies, showed improvements in BMI after 6months of 10.5g / day of either psyllium or guar fiber supplementation compared with a standard diet in hypertensive, slightly overweight individuals. Similarly, in a randomized controlled trial, Abutair et al. (2016) found that in 40 overweight and obese T2DM patients after 8-week intervention with 10.5g of psyllium added to their habitual diet, the supplement reduced weight, BMI, waist circumference, and hip circumference. The anti-obesity effects were confirmed by Pal et al. (2016) that conducted a long-term randomized, placebo-controlled trial with overweight and obese adults. These subjects were treated with 5g of psyllium supplement added to the usual diet 3 times. After 52-week intervention, significantly reduction of waist circumference, body fat mass, and significantly increase of lean mass were observed.

1.6.6 Diabetes, obesity and gut microbiota

In the past few years, there has been a surge in microbiome research and the focus has begun to shift from correlational studies towards mechanistic and clinical studies in understanding how the microbiome is able to influence human health and disease progression (Fischbach, 2018).

Recent evidence suggests that the microbiome may affect the probability of many major diseases, including obesity and diabetes. The potential role of the gut microbiome in these metabolic disorders has been identified (Hartstra et al., 2015).

Targeted metagenomics studies have revealed that approximately 90% of the bacterial species present in the gut of adults belongs to phyla *Bacteroidetes* (Gram-negative) and *Firmicutes* (Gram-positive) (Sharma & Tripathi, 2019). A healthy adult harbor 500–1000 bacterial species at a time and there can be 1012–1014 colony-forming units (CFU) in the whole gut with a mass weight of about 1–2 kg. The colon alone contains approximately 109–1012 CFU/ml followed by 101–103 CFU/ml in jejunum and 104–108 CFU/ml in the ileum (Sharma & Tripathi, 2019).

Gut microbiota is involved in several intestinal biological functions, such as the defence against pathogens, immunity, the development of the intestinal microvilli and the degradation of non-digestible polysaccharides (Delzenne et al., 2011).

In physiological condition, the composition and the activity of the gut microbiota is stable. The gut barrier function is maintained via several mechanisms such as the appropriate localization and distribution of tight junction proteins (claudin, zonula occludens-1 and occludin), a normal endocannabinoid system tone and lipopolysaccharide detoxification by intestinal alkaline phosphatase. Altogether, these factors contribute to the maintenance of appropriate energy, lipid and inflammatory homeostasis (Everard et al., 2013).

In T2DM and obese patients, was observed gut barrier alterations leading to disruption in the gut microbiota-host symbiotic relationship. This increase in gut permeability results from different disturbances:

a) alterations in the gut microbiota composition and/or activity. In particular, human studies reported changes in the abundance of the principal bacterial phyla in type 2 diabetic patients while, the increase in the *Firmicutes/Bacteroidetes* ratio in obese patients remains a matter of debate (Ley et al., 2016; Wu et al., 2010; Schwiertz et al., 2010). The *Bifidobacterium* abundance appears to be lower in overweight, obese or type 2 diabetic patients than in lean subjects (Wu et al., 2010; Schwiertz et al., 2010). Another species decreased by type 2 diabetes is *Faecalibacterium prausnitzii* (Furet et al., 2010).
b) alterations in the expression, localization and distribution of tight junction proteins leading to an increase in paracellular gut permeability. Gut barrier alterations are responsible for metabolic endotoxaemia (increase plasma LPS levels) leading to low-grade inflammation and metabolic disorders such as alterations of glucose and lipid homeostasis (Everard et al., 2013).
c) overactivation of the cannabinoid receptor 1 (CB1). The endocannabinoid system is involved in the regulation of gut barrier function in obesity (Muccioli et al., 2010). This system is composed of endogenous bioactive lipids that acted by activating two G protein-coupled receptors: cannabinoid receptor 1 (CB1) and 2 (CB2), expressed throughout the gastrointestinal tract (Alhouayek et al., 2012). The endocannabinoid lipids, anandamide and 2-arachidonoylglycerol are CB1 and CB2 ligands, with anandamide having a greater affinity for CB1, involved in the regulation of gut barrier functions (Alhouayek et al., 2011). However, the gut barrier regulation in obesity appears to be CB1 dependent, as CB1 antagonists

decrease gut permeability and metabolic endotoxaemia in genetically obese and type 2 diabetic mice (Muccioli et al., 2010). Moreover, the endocannabinoid system exerts these effects on epithelial permeability by altering the distribution of tight junction proteins (Muccioli et al., 2010). Thus, the gut microbiota could induce their effects on gut barrier alterations and metabolic endotoxaemia through the endocannabinoid system in the pathology of obesity and DMT2.

d) a decrease in intestinal alkaline phosphatase (IAP) activity leading to a decrease in LPS detoxification. Among mechanisms implicated in enabling the symbiotic relationship between the gut microbiota and the host, is also the action of IAP. This enzyme is involved in the breakdown of dietary lipids but, it also plays an important role in LPS detoxification by dephosphorylating the lipid portion of the LPS (Koyama et al., 2002). The expression of IAP is controlled by the gut microbiota (Bates et al., 2007). The obesity appears to be associated with a decrease in IAP activity even though the IAP activity (Lalles et al., 2010). Thus, modifications of the IAP activity by a high-fat diet and gut microbiota contribute to the alterations in the gut barrier functions in obesity and type 2 diabetes.

Prebiotic treatment restores these alterations in the gut microbiota, modulates enteroendocrine peptides and improves gut permeability these changes are associated with a reduction in low-grade inflammation and the restoration of glucose and lipid homeostasis in obesity and type 2 diabetes (Everard et al., 2013).

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Chapter 2
Citrus × *clementina* Hort.: chemistry and bioactivity

2.1 The *Citrus* genus

Citrus is the most produced tree fruit crop in the world. The increase of *Citrus* world production is constant in XXIth century, and annual production has reached more than 131.3 million tons (FAO 2015). Oranges represented the major one with 61% of the *Citrus* production, followed by mandarins (20%), limes and lemons (14%), and grapefruits (5%). Brazil was the main producer followed by Mediterranean countries, China and the USA. There are two different markets: the fresh fruit and the processed juice market. In the Mediterranean basin, these fruits are primarily produced for the fresh juice market.

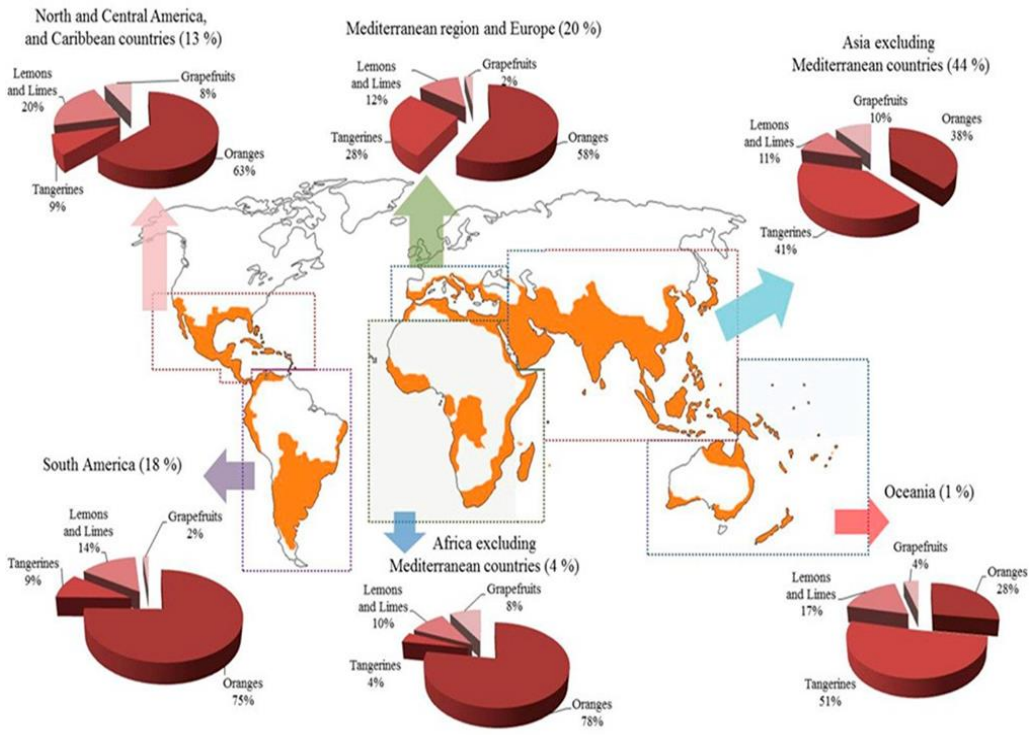


Fig. 1. Climate sustainability and the annual production of citrus fruits in different geographical regions across the globe. (Adapted from Mahato et al., 2019).

Almost 33% of the *Citrus* fruits are industrially processed for juice production, however, a great amount of *Citrus* wastes including peels, segment membrane and seeds are produced (Figure 1). A worldwide production of 15 million tons *per* year of *Citrus* wastes was estimated. Due to the low cost and easy availability, the residues of *Citrus* fruit, discarded as waste in the environment, should be considered as potential nutraceutical source. In fact, these by-products are rich in bioactive phytochemicals, and

could be recycled as value-added food supplements, which provide advantageous dietary fibers, polyphenols and other bioactive compounds (Mahato et al., 2019).

Citrus species are diploids (2 n = 2 x = 18). The trees are greenish of different sizes and heights, depending on the species and produce fruits of various sizes and form, oblong and round. The fruit is covered and protected by green or yellow epicarp composed of glands that contain essential oils (EO), responsible for the peculiar fragrance. The epicarp include also a white, spongy and thick mesocarp which together with the epicarp forms the pericarp or peels of the fruit. Inside the fruit is divided into cavities in which there are sacs of juice including seeds depending on the species. *Citrus* species easily hybridize and new hybrids are continuously developed by cross-pollination to obtain the desired qualities like juicy, seedless, and fresh fruit (Alexander, 2019).

The genus *Citrus* and related genera (*Fortunella*, *Poncirus*, *Eremocitrus* and *Microcitrus*) belong to subfamily Aurantioideae of the Rutaceae family, which is widely distributed across the monsoon region from west Pakistan to north-central China and south through the East Indian Archipelago to New Guinea and the Bismarck Archipelago, northeastern Australia, New Caledonia, Melanesia and the western Polynesian islands. This genus is defined by two different classification systems: Tanaka's, with 156 species, and Swingle's, with only 16 species. Major horticultural *Citrus* groups such as the orange (*C. sinensis* (L.) Osb.), mandarin (*C. reticulata* Blanco), lemon (*C. limon* (L) Burm.), grapefruit (*C. paradisi* Macf.), lime (*C. aurantifolia* (Christm.) Swing.) and pummelo (*C. maxima* (Burm.) Merr.), are each considered as species in Swingle's systematics. This controversial situation results from the conjunction of a broad morphological diversity, the overall sexual interspecific compatibility within the *Citrus* genus and between genera, and the partial apomixis of many cultivars (Ollitrault & Navarro, 2012).

The classification of Swingle and Reece distinguishes the Eucitrus subgenus, where all the cultivated taxa are found, and the subgenus Papeda. The subgenus Padela, in contrast to Eucitrus, is characterized by:

- bitter taste of the pulp that makers fruits inedible
- wings of the perioles are markedly broad, in some case being larger that blades
- stameus are free from the base insteade of fused
- flowers and new growth are purple
- the juice vesicles, inside of the carpels, are adhered either to the external part of the membrane or to the lateral sides. In Eucitrus, they are always adhered to the external part.

The subgenus Eucitrus include *C. aurantium*, *C. reticulate*, *C. sinensis*, *C. limon*, *C. autantifolia*, *C. grandis*, *C. medica*, *C. paradisi*, *C. indica* and *C. tachihana* (Dugo & Di Giacomo, 2004)

2.1.1 State of art of *Citrus* bio-activity

Traditionally, *Citrus* varieties have been used worldwide as medication in the treatment of several diseases. The potential health promoting effects of *Citrus* consumption includes antioxidant, anti-hypercholesterolemic, anti-diabetic, antiulcer,

anti-inflammatory, anti-cholinesterase and anti-platelet activities (Xiang et al., 2017; Gabriele et al., 2017).

Antioxidant activity

Reactive oxygen species (ROS) and nitrogen species (RNS) are involved in the pathogenesis of many human diseases and antioxidants play a crucial role in restoring the physiological oxidative balance and modulating biological pathways and membrane function (Smeriglio et al., 2018). The *Citrus* genus are recognized for their protective effects against free radical-induced damage in oxidative stress directed on target biomolecules. Smeriglio et al. (2018), reported the dose-dependent antioxidant activity of *C. lumia* EO with IC_{50} values of 22, 46, 104, and 233 μg/mL for β-carotene bleaching test, oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric reducing antioxidant power (FRAP) and trolox equivalent antioxidant capacity (TEAC) assay, respectively. Previously, Bonesi et al. (2017) investigated six *Citrus* petitgrain essential oils (EO) for their antioxidant properties. In this study, *C. aurantium* petitgrain oil demonstrated the strongest radical scavenging activity in DPPH assay with an IC_{50} value of 27.2 μg/mL, followed by *C.* × *clementina* oil with an IC_{50} value of 39.0 μg/mL. While, in β-carotene bleaching test the highest antioxidant capacity was observed with *C. sinensis* oil with IC_{50} values of 176.3 and 51.3 μg/mL after 30 and 60 min of incubation, respectively. The results were in according with Tavena et al. (2019) that demonstrated EO from *C. aurantium* L. showed the highest antioxidant activities valued 88.1% in DPPH assay. Conversely, Choi et al. (2000) analysed four EOs from *C. aurantium* L. and found scavenging effects in the range from 17.7 to 34.1%. These differences would be explained by the different chemical compositions of the same *Citrus* species in different regions.

Haraoui et al. (2019) compared the antioxidant activity of leaf extracts and juices of ten varieties of *Citrus* fruits. All investigated samples exhibited radical scavenging activity with IC_{50} values in the same order of positive controls ascorbic acid and BHT. Among them, *C. maxima* and *C. aurantium* juice and leaves showed the highest DPPH radical scavenging activity with IC_{50} values of 0.42 and 0.44 mg/mL, and 051 and 0.57 mg/mL, respectively. The same trend was observed also in β-carotene bleaching test with percentage exceed 80.55%, followed by Sanguinelli and limon.

More recently, Smeriglio et al. (2019), revealed that the *C. lumia* albedo extract showed marked antioxidant and free radical scavenging properties depending on the reducing ability of hydroxylated phenolic structures and glycosylation grade. This extract showed IC_{50} values of 2.57 and 5.22 μg/mL in ORAC and FRAP assay, while in TEAC, DPPH and β-carotene bleaching assay it showed less activity with IC_{50} values of 73.19, 533.03, and 118.21 μg/mL, respectively. Ali et al. (2019a) analysed the methanolic extract of pomelo and found IC_{50} value of 1.70 μg/mL in DPPH radical scavenging assay and 1848.2 μM Fe(II)/100g in FRAP test. According to García-Salas et al. (2013) that investigated the antioxidant potential of whole-lemon powder, Gabriele et al. (2017) tested the potential antioxidant capacity of bergamot extract and found an ORAC value

of 950 units per g of powder. In addition, in DPPH test an EC_{50} value of 720 mg/mL was found. This value is greater than that reported by Trombetta et al. (2010) for two extracts of bergamot peels. Furthermore, the bergamot extract showed a higher 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonico) (ABTS) radical inhibition with value of 136.3 mmol Trolox Equivalent/g dry weight (mmol TE/g DW). Menichini et al. (2016) demonstrated the capacity of *C. medica* Diamante peels extract to inhibit both DPPH and ABTS radical in a concentration-dependent manner with IC_{50} values of 0.81 and 3.48 mg/mL, respectively. In β-carotene, this extract exhibited an IC_{50} value of 0.23 mg/mL while, in the FRAP test exhibited a limited ability with value of 3.88 mM Fe(II)/g.

More recently, the antioxidant activity of methanol leaves extract and ethyl acetate fraction of *C. pseudolimon* were examined by Kumar et al. (2019). The ethyl acetate fraction displayed greater DPPH radical scavenging activity than the methanol leaves extract with IC_{50} values of 278.60 and 313.20 μg/mL, respectively. The IC_{50} values of 476.39 and 498.26 μg/mL were found in H_2O_2 scavenging assay. The antioxidant effects of ethanol extract and its three subfractions, petroleum ether, ethyl acetate, and water extracts, from *C. sinensis* Osbeck cv. Gannanzao peels, were evaluated by Long et al. (2017). The ethyl acetate extract exhibited the best antioxidant potential compared to four extracts in all antioxidant assay with IC_{50} value of 38.33, 8.47 μg/mL in DPPH and ABTS test, respectively and values of 21.54 μM TE/mg DW in FRAP assay.

The antioxidant activity of *C. limon* L. Burm. cv Femminello comune juice was analysed by Loizzo et al. (2019) and found IC_{50} values of 40.3 and 46.5 g/mL in DPPH and ABTS test and 49.7 mg Fe(II)/g in FRAP test. Favela et al. (2016) demonstrated that Moro juice had ABTS and DPPH radical scavenging activity with TEAC values of 14.30 and 14.39 μM, respectively. Previously, Gironés-Vilaplana et al. (2014) analysed the lemon, orange, grapefruit, lime, and mandarin fruits for their antioxidant activity. Lemon and grapefruit exhibited the highest scavenging activity while orange the lowest in DPPH test. A similar trend was observed in ABTS assay. Abirami et al. (2014) reported the ABTS scavenging activity of *C. maxima* (red), *C. hystrix* and *C. maxima* (white) juice with TEAC values in the range of 1,343.90 and 34,659.62 mmol TE/L juice. The oxidation of β-carotene was moderate inhibited by all juices (56.99-62.18%).

Anti-diabetic activity

Diabetes is characterized by high concentrations of blood sugar, which can cause serious complications. The treatment of diabetes therefore mainly focuses on reducing fluctuations in blood sugar. The α-amylase and α-glucosidase inhibitors are currently used for diabetic treatment as oral hypoglycaemic agents. Acarbose, is a commercially drug largely used in T2DM. However, it is reported to cause various side effects such as abdominal distention, flatulence and possibly diarrhoea (Loizzo et al., 2018). Searching for safe and effective carbohydrates-hydrolysing enzymes inhibitors from natural sources are of emerging interest. Recently, the hypoglycaemic activity of methanol leaves extracts and ethyl acetate fraction of *C. pseudolimon* was examined by Kumar et al. (2019). The ethyl acetate fraction displayed a greater inhibition against α-glucoside (84.18%) in

comparison to the methanol extract (82.94%). IC_{50} value of 83.66 and 78.52% for ethyl acetate and methanol extracts, respectively were found against α-amylase. Basli et al. (2016) tested the capacity of *C. lemon* fruit extract to inhibit α-amylase with IC_{50} value of 103.46 µg/mL. The potential inhibition of *C. maxima* (red), *C. maxima* (white) and *C. hystrix* juices against α-amylase was investigated by Abirami et al. (2014) that found percentage in the range 75.55-79.75%. A similar trend was observed against α-glucosidase in which the following potency was observed *C. maxima* (red) > *C. maxima* (white) > *C. hystrix*. Oboh et al. (2017) suggested that the lemon peels EO exhibited stronger inhibitory activity on α-amylase and α-glucosidase activities (IC_{50} values of 8.16 and 7.56 µg/mL, respectively) compared to orange peels EO (IC_{50} values of 11.51 and 11.53 µg/mL, respectively).

Previously, Uddin et al. (2011) demonstrated that *C. macroptera* fruit extract inhibited α-amylase with value of 3.64 mg/mL. The peels of four pomelo varieties (*C. grandis*) were investigated as potential source of phytochemicals useful for the treatment of metabolic disorders (Ding et al., 2013). Liangpinyou (LP) and beibeiyou (BB) varieties extracts were more potent in anti-metabolic disorder effects than the duanshiyou (DS) and wubuyou (WB). Indeed, diet supplementation with 1% (w/w) for 8 weeks in C57BL/6 mice of LP and BB blocked the body weight gain, lowered fasting blood glucose, serum TC, liver lipid levels, and improved glucose tolerance and insulin resistance, and lowered serum insulin levels in HF diet-fed mice. Compared with the HF group, LP and BB peels extracts increased the mRNA expression of PPARα and its target genes, such as FAS, PGC-1α and PGC-1β, and GLUT4 in the liver and white adipocyte tissue.

Kumar et al. (2019) indicated that oral administration of methanol leaves extract (200 mg/kg) and ethyl acetate fraction (100 mg/kg) of *C. pseudolimon* for 21 days decreased the fasting blood glucose level in diabetic rats. Previously, Sathiyabama et al. (2018) reported that the administration of doses of 50 and 100 mg/kg of *C. sinensis* peels extracts in diabetic rats reduced both fasting blood glucose by 56.1% and 55.7%, respectively and plasma insulin levels by 22.9% and 32.7%, respectively. The administration of 600 mg/kg of *C. medica* Diamante peels extract in db/db rat, significantly decreased the serum glucose (Menichini et al. 2016). The *in vivo* reduction of blood glucose level and plasma insulin level was demonstrated for both *C. reticulata* and *C. Sudachi* peels extract (Guo et al., 2016; Kobayashi et al., 2017).

Recently, Ye et al. (2018) revealed that *Citrus* albedo extract can be used in preparation of drug, health products or food for treatment or prevention of T2DM (Patent no. CN108379372A).

Anti-obesity activity

Pancreatic lipase inhibition is one of the most largely studied mechanisms of the anti-obesity activity of natural products. The inhibition of this enzyme delays the digestion of triglyceride to absorbable free fatty acids with reduction of postprandial hypertriacylglycerolemia.

Recently, the anti-lipase activity of *C. reticulata* extracts collected at different times was evaluated (Zeng et al., 2018). These extracts were able to inhibit the lipase enzyme in a concentration-dependent manner and showed IC_{50} values in the range between 0.383 and 1.370 mg/mL. Basli et al. (2016) reported that lemon extract inhibited lipase with IC_{50} value of 110.07 μg/mL. Moreover, *C. unshiu* peels extract showed inhibitory effect on lipase activity with IC_{50} value of 507.01 μg/mL (Kim et al., 2016a). Previously, IC_{50} values in the range of 0.25-0.36 mg/mL were found for the pulp extract of the six pomelo cultivars against pancreatic lipase (Makynen et al., 2013).

More recently, Kamel et al. (2019) tested the potential health benefits of *C. reticulate* peels water extract. The supplement (800 mg of dry extract/day) was administered for ten days to obese patients. Clinical trials showed a reduction in body mass index, body fat percentage and in waist circumference, by 5.74 kg/m^2, 4.24 % and 11.33 cm, respectively.

Dietary ingestion of *C. tumida* Hort. ex Tanaka peels (5% w/w) significantly suppressed body weight gain by decreasing epidydimal, perirenal, and subcutaneous fat weights (Sato et al., 2019). A similar effect was observed also with *C. aurantium* extract (100 mg/kg/day) that administered for eight weeks in high fat diet-induced obese C57BL/6 mice determined a significant decrease of body weight. Additionally, the same treatment in 3T3-L1 adipocytes determined a reduction of lipid droplets regulating adipogenesis and thermogenesis *via* AMPKα pathway (Park et al., 2019).

Kegele et al. (2019) investigated the effects of CitrusiM® (*C. sinensis* dried extract) on body composition: percentage of lean mass and percentage of fat mass. This extract, determined a significant reduction of fat, increase lean mass reducing waist circumference after doses of 0.5 or 1 g/day. Female C57BL/6 mice were fed with diet supplemented with 1% w/w citrange peels extract or 1% w/w citrange flesh and seed extract for 8 weeks. Results showed that both extracts regulated the glucose metabolic disorders in obese mice. Indeed, the body weight, blood glucose, serum total cholesterol and low-density lipoprotein cholesterol levels were significantly reduced. These effects were probably due the inhibition of PPARγ and LXRs gene expressions (Favela-Hernández et al., 2016). The obese mice treated with supplementation of 0.25 and 0.5% of *C. reticulata* extract in food for 12 weeks exhibited reductions of 21 and 34%, in body weight in mice, respectively (Guo et al., 2016). Moro juices (*C. sinensis*) extract (Morosil®, 400 mg/die) was able to induce a significant reduction in body mass index (BMI) after 4 weeks of treatment (Cardile et al., 2015). The anti-obesity effects of *C. sunki* peels extract *in* C57BL/6 obese mice and mature 3T3-L1 adipocytes was also demonstrated. This extract increased of β-oxidation and lipolysis in adipose tissue (Seong-Il et al., 2012).

Hypocholesterolaemic effects

Literature data showed that *Citrus* genera possesses beneficial properties related to cholesterol (Favela et al., 2016). More recently, Kamel et al. (2019) tested the potential benefits of the water extract of *C. reticulata* peels after supplements with 800 mg of dry extract/day for ten days and observed a reduction of total cholesterol and triglyceride by 35.56 and 24.66 mg/dL, respectively.

Fayek et al. (2017) investigated the hypocholesterolaemic effects of mandarin peels aqueous and *n*-hexane extracts. The results showed that these extracts decrease the cholesterol level by 59.3 and 56.8%, respectively. The potential effects of a phytocomplex from bergamot fruit as dietary supplement was evaluated by Di Folco et al. (2018). After 6 months of administration, a significant reduction of total cholesterol (-15%), LDL-cholesterol (-22%), triglycerides (-23%), and a significant increase in the HDL cholesterol (+ 14%) were observed. The administration of lyophilized *C. sinensis* juice at a dose of 5 g/kg for 15 days on adult male Wistar rats decreased plasma levels of cholesterol, LDL and triglycerides by 31, 44 and 33%, respectively. In addition, microsized insoluble fibers from *C. sinensis* fruits reduced the concentrations of serum triglycerides of 15.6-17.8%, the serum total cholesterol of 15.7-17.0% through an upgrade of cholesterol excretion (123-126%) and bile acids in feces (Favela et al., 2016).

Makynen et al. (2013) suggested that the pomelo extracts may help to reduce blood cholesterol and triglycerides by inhibiting of pancreatic cholesterol esterase ranging from 1.50 to 2.90 mg/mL. A reduction in cholesterol and triglycerides levels were observed also with *C. medica* cv Diamante peels hydroalcoholic extract of (300 and 600 mg/kg/die) administered in Zucker diabetic rats for 4 weeks (Menichini et al., 2016).

Previously, Kang et al. (2012) reported a significant reduction in body weight gain, adipose tissue weight, serum total cholesterol, and triglyceride after dietary supplementation with *C. sunki* peels extract in obese C57BL/6 mice.

Anti-inflammatory activity

Nitric oxide (NO) is recognized as a mediator and regulator in pathological reactions, especially in acute inflammatory responses (Terao, 2009). The development of substances to prevent the overproduction of NO has become a new research target to treat chronic inflammatory diseases. *C. unshiu* peels extracts were investigated on the anti-inflammatory activities in murine macrophages and moisturizing effects in human keratinocytes (Kim et al., 2019a). Results evidenced that *C. unshiu* peels extracts are able to suppress lipopolysaccharide (LPS)-induced NO without exerting cytotoxic effects on RAW 264.7 cells. Moreover, extracts inhibited the expression of inducible nitric oxide synthase (iNOS), COX-2 protein, tumour necrosis factor (TNF)-α and interleukin (IL)-6. Similarly, *C. maxima* peels aqueous extract inhibited the secretion of inflammatory cytokines IL-6 and TNF-α in LPS-stimulated RAW264.7 cells without cytotoxicity (Ishida et al., 2019).

Flowers and leaves extracts of *C. medica* cv Diamante showed inhibition of LPS-induced NO production in RAW 264.7 cells in a dose-dependent manner, with an IC_{50} values of 525.0 and 574.0 μg/mL, respectively (Menichini et al., 2011). After the administration of high dose of C. *sinensis* extract, the NO content decreased by more than 60% (Pantsulaia et al., 2014). *C. sunki* immature and mature peels extracts exhibited IC_{50} values of 188.4 and 580.9 mg/mL, respectively on NO production in LPS-Activated RAW 264.7 cells (Choi et al., 2007). Previously, Impellizzeri et al. (2015) reported the *in vivo* anti-inflammatory activity of bergamot juice extract. Mice treated with this extract

were more resistant to induction of colitis and a reduction in the expression of important inflammatory mediators, TNF-α and interleukin 1 β (IL-1β), was observed.

Antiproliferative activity

C. reticulata cv. suavissima flavedo extract exhibited a potential anti-tumour activity due its inhibitory effect on epithelial-to-mesenchymal transition due the ability to interfere with the canonical TGF-β1-SMAD-Snail/Slug axis (Chang et al., 2015). *C. reticulate* peels extract showed antiproliferative effects on hepatic and mammary carcinogenesis by inducing apoptosis *via* p53 expression (Meiyanto et al., 2012). A promising activity against mammary carcinoma was demonstrated also by *C. aurantifolia* peels extract that inhibited human breast adenocarcinoma (MCF-7) cells growth and proliferation of cancer in female Sprague dawley rat by inducing a down-regulation of c-myc (Adina et al., 2014). *C. sinensis* cv. Washington Navel and cv. Sanguinello juices were investigated for their antiproliferative activity. The juice of fruits of *C. sinensis* cv. Washington Navel and cv. Sanguinello at concentrations of 82.6% and 73% showed 100% antiproliferative activity against human chronic myelogenous leukemia (K562) and human leukemia (HL-60). In the same way at concentration of 10% the 90.5% of antiproliferative activity was observed against MCF-7 cells (Camarda et al., 2007).

Antibacterial activity

The antibacterial activity of *C. limon* and *C. aurantium* varieties, juice and leaves extracts, was investigated by Haraoui et al. (2019). The juice was more active than the leaves. In particular, *C. limon* juice exhibited inhibition zone of 27.66 mm on *Micrococcus luteus* followed by *C. aurantium* with areas of 24.66 cm against *Staphylococcus aureus.* Interesting results were observed also for *C. maxima* and *C. clementine* with inhibition zone of 23.00 and 17.66 mm, respectively against *M. luteus*.

Gabriele et al. (2017) reported the antimicrobial activity of bergamot extract against potentially pathogenic bacteria. This sample was most active against gram negative microorganisms such as *Escherichia coli* and *Salmonella typhimurium* with minimum inhibitory concentration (MIC) values of 500 mg/mL, whereas *Enterobacter aerogenes* was inhibited only at 1000 mg/mL. Concerning to gram-positive bacteria, *S. aureus* was more sensitive than *Enterococcus faecalis* to the action of bergamot phytocomplex with MIC values of 500 and 700 mg/mL, respectively.

In agreement with Gabriele et al. (2017), gram-positive bacteria were more sensitive to the action of the oil with inhibition zones being between 9 and 12.5 mm. More recently, the antimicrobial activity of the essential oil of *C. aurantium* was demonstrated against *S. aureus*, *Salmonella* sp., *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *E. coli* (Taneva et al., 2019). Sweet orange oil and its major constituents decanal, octanal, and linalool showed bactericidal effects on *E. coli*, *S. aureus*, *Saccharomyces cerevisiae* and *Aspergillus niger* with MIC values in the ranges 25-100, 50-100, 6.25-100, and 50 μg/mL, respectively (Liu et al., 2012). A mixture of *C. sinensis* and *C. bergamia* EO showed inhibitory activity with a MIC values ranging from 0.25 to 0.5% (v/v) and a

minimum inhibitory dose (MID) of 50 mg/L against *Enterococcus faecium* and *E. faecalis*, respectively (Fisher & Phillips, 2009). In addition, *C. sinensis* EO had strong antibacterial activities on *Listeria monocytogenes*, *E. coli*, *Salmonella enteritidis*, *Proteus mirabilis* and *Bacillus cereus* (Irkin, 2009).

Neuroprotective activity

Neuropsychological alterations caused by diseases such as Alzheimer's disease (AD) are attributed in part to cholinergic transmission disorders. The most common treatments for AD and related dementia are acetylcholinesterase inhibitors, some of which are derived from natural sources such as galantamine (Xiang et al., 2017). Recently, Smeriglio et al. (2018) reported the role of *C. lumia* EO against acetylcholinesterase enzyme (AChE) with an IC_{50} value of 258.25 μg/mL. This activity is about 3-times higher than that previously reported for *C. limon* EO (IC_{50} value of 849.90 μg/mL) (Aazza et al., 2011), but in agreement with Tundis et al. (2012) that reported the AChE inhibitory activities of *C. aurantifolia*, *C. aurantium*, and *C. bergamia* EO (IC_{50} values of 139.3, 147.5 and 161.5 μg/mL). Abirami et al. (2014) reported the strong AChE inhibitory activity of *C. hystrix*, *C. maxima* (red) and (white) juices with values of 75.71 and 79.74%, respectively.

Citrus and derived compounds: gut microbiota interaction

The interaction between the gut microbiota, a complex ecosystem that varies between individuals, and polyphenols is considered to be bidirectional to the capacity of intestinal bacteria to metabolize polyphenols. Polyphenols may induce a positive change in the microbiota regarding composition and activity, such as the production of short-chain fatty acids (SCFAs) in the colon. Additionally, the metabolites have many known beneficial biological effects, acting as fuel for enterocytes, improving barrier function, and inhibiting inflammation (Rios-Covian et al., 2016; Van der Beek et al., 2017).

Ingestion of bioactive compounds, such as hesperidin and naringin, found in *Citrus* fruits and juice, can improve the homeostasis of gut microbiota. There is growing scientific interest in the effects of functional foods and nutraceuticals on human health with particular reference to gut microbiota.

Lima et al (2019) suggested a prebiotic effect of orange juice as an alternative healthy drink, with the positive effect on the intestinal microbiota and metabolic biomarkers of young women. The results showed that daily intake of orange juice (300 mL/day for 2 months) did not change women's body composition, but improved blood biochemical parameters, such as low-density lipoprotein-cholesterol, glucose, and insulin sensitivity. Additionally, orange juice positively modulated the composition and metabolic activity of microbiota, increasing the population of faecal *Bifidobacterium* spp. and *Lactobacillus* spp and reduction of Enterobacteria. A reduction of ammonium ($NH4^+$) and an increase of the production of short-chain fatty acids (SCFAs) were also demonstrated. SFCAs are recognized as essential for gut physiology because they function as a primary source of energy to the enterocytes, stimulate epithelial cell proliferation, improve blood flow, and

promote the absorption of sodium and water that can stop diarrhoea, decrease the intraluminal pH and, finally, decrease the absorption of ammonia. Indeed, after 30 days of orange juice, the female volunteers showed a significant increase of butyric acid and acetic acid. The increase in acetic acid production is beneficial because it may downregulate the cholesterol synthesis after absorption (Alvaro et al., 2008) and promotes calcium absorption in the colon (Donatto et al., 2006). The increases of butyric acid is beneficial, since it is the main energy source of the colonocytes and has anti-inflammatory and anticarcinogenic properties (Wong et al., 2006).

More recently, Fidélix et al. (2020) confirmed the prebiotic effect of orange juice on intestinal microbiota and a positive influence on metabolic biomarkers on ten healthy female volunteers after intervention with 300 mL/day orange juice for 60 days. Orange juice stimulated the increase of *Lactobacillus* spp. in the intestinal microbiota and improve glucose metabolism.

Daily supplementation of two oranges (cv Cara Cara and cv Bahia) juices with different flavanone content for 7 days in healthy volunteers resulted in increase in the abundance of Lachnospiraceae and Ruminococcaceae that represented the most abundant Firmicutes families present in the gut environment (Brasili et al., 2019).

The reduction in the relative abundance of Lachnospiraceae members in the gut microbiota was correlated with compromised health status of patients suffering from colorectal cancer, ulcerative colitis, and type 2 diabetes (Brasili et al., 2019). While, the importance of Ruminococcaceae family for the gut health is indicated by their reduced abundance in faeces of Crohn's disease and ulcerative colitis patients (Rajilić-Stojanović et al., 2013). Additionally, Lachnospiraceae and Ruminococcaceae are recognized for capacity to generate SCFAs, mainly butyrate from fermentation of nondigestible plant fibers (Vital et al., 2014). Interestingly, after Cara Cara juice intake positive correlations were found between Lachnospiraceae family and butyrate, as well as among the most abundant SCFAs present in the colon, including acetate, butyrate, and propionate (Brasili et al., 2019).

The effect of hesperidin supplementation on microbiota composition and SCFAs was investigated by Steven et al. (2019). In this randomized, placebo-controlled trial in healthy subjects with features of metabolic syndrome, daily supplementation with 500 mg citrus extract (with >80% hesperidin-2S and >4% naringin) for 12 weeks, did result in an increase in the butyrate to total SCFA ratio but not in the absolute levels of faecal SCFAs. Additionally, supplementation with this citrus extract reduce levels of faecal calprotectin, used as biomarker of intestinal inflammation.

Citrus and derived compounds: drug interaction

Many drug-food interactions tend to occur due to the concomitant use of drugs and dietary habits, food composition, and dietary supplement (Boullata et al., 2012). Food and drug interactions are defined as an alteration in pharmacokinetics or pharmacodynamics of a drug. Pharmacokinetics refers to the quantitative description of drug disposition, which includes absorption, distribution, metabolism, and excretion while

pharmacodynamics refers to the physiologic or clinical effects of a drug (Chan, 2002). Changes in pharmacokinetic parameters, such area under the concentration-time curve (AUC), maximum plasma concentration (Cmax), time to reach Cmax (Tmax) and elimination half-life (t1/2) of drug, resulting from ingestion of combinations of food, would depend on the kind and quantities of foodstuffs ingested, the drug itself and the timing administration relative to food intake (Saito al., 2005).

Bobroff et al. (2009) suggested that food-drug interactions are frequently determined by chelating with food element that may influence the bioavailability of certain drugs. Chan et al. (2006) also reported that food-drug interactions can result in two main clinical effects:

- decrease bioavailability of a drug, which predisposes to treatment failure;
- increase bioavailability, which increases the risk of adverse effect and may even induce toxicities.

Patients who presents increase risks of adverse effects associated with drug-nutrient interactions are elderly patients, cancer patients, malnutrition patients, gastrointestinal tract dysfunctions, acquired immunodeficiency syndrome and those receiving enteral nutrition (Yadav et al., 2020).

Various enzymes are involved in drug metabolism and food may alter the hepatic metabolism of some drugs. Cytochrome P-450 (CYP) is a multigene family of heme containing proteins found in the endoplasmic reticulum of cells throughout the body. The largest concentrations of these enzymes are located in the liver and the intestinal wall, where they play a role in the oxidative biotransformation of numerous endogenous substances and xenobiotics. Several isoforms have been distinguished based on their structure, substrate specificity, or responses to various types of inducers. The CYP3A sub family represents the predominant and most abundant enzyme group. In the liver, CYP3A comprises on average 30% of all CYP content and as much as 70% in enterocytes, in small intestine epithelial cells (Zhang et al., 1999). Located in the apical brush border of the enterocytes is the P-glycoprotein (Pgp) membrane transporter, a member of the ABC (adenosine triphosphate-binding cassette) superfamily of proteins. The role of the Pgp transporter is to carry lipophilic molecules from the enterocyte back into the intestinal lumen. After uptake by the enterocyte, many lipophilic drugs are either metabolized by CYP3A4 or pumped back into the lumen by the Pgp transporter. Therefore, CYP3A4 and Pgp may act in tandem as a barrier to oral delivery of many drugs (Kane & Lipsky, 2000). In addition, Saito et al. (2005) suggested that also the Organic Anion Transporting Polypeptide (OATP) is involved in transporters in the small intestine.

Grapefruit juice and food interactions have been well documented by various authors in the past two decades (Kane & Lipsky, 2000; Yadav et al., 2020). Studies reported a pronounced effect of grapefruit juice on intestinal cytochromes P450 system and less effect at the hepatic level (Flanagan, 2005). Grapefruit and their components can affect CYP450 drug oxidation and transportation and inhibit cytochrome CYP3A4 in the small intestine causing significantly decreased first-pass metabolism, which results in increased

drug absorption and systemic drug bioavailability (Yadav et al., 2020). The drug-grapefruit juice interactions were observed on:

- ✓ Calcium channel antagonists such as felodipine, nisoldipine, amlodipine, verapamil, diltiazem. The juice determined inhibition of CYP3A4, CYP1A2, P-glycoprotein and increased bioavailability with consequent hypotension and tachycardia (Sica, 2006);
- ✓ Central Nervous System (CNS) modulators such as: diazepam, triazolam, midazolam, alprazolam, carbamazepine, buspurone sertraline. The juice inhibited CYP3A4, CYP1A2, MRP2, OATP-B and P-glycoprotein and increased bioavailability with increased CNS and depression (Pawełczyk & Kłoszewska, 2008);
- ✓ Statins such as simvastatin, lovastatin, atorvastatin, pravastatin. Furanocoumarins of grapefruit juice inhibited the metabolism of statins and increased adverse effects (Reamy & Stephens, 2007). These effects were observed after of 1.200 mL per day. Even patients who enjoy grapefruit juice do not ingest more than one quart each day. In contrast, another study (Lilja et al., 2000) examined normal-dose lovastatin (40 mg) in patients who consumed a typical amount of grapefruit juice. This study showed a minimal effect on plasma concentrations of lovastatin and no deleterious clinical outcomes;
- ✓ Immunosuppressants such as cyclosporine. By randomized crossover design, 18 healthy volunteers received cyclosporine (5 mg/kg) with 240 mL orange juice. The juice inhibited CYP3A4, P-glycoprotein and increased bioavailability with increase of adverse effect (Paine et al., 2008) such as nephrotoxicity, hypertension and cerebral toxicity (Saito et al., 2005);
- ✓ Antivirals such as saquinavir. The juice inhibited CYP3A4, CYP1A2, MRP2, OATP-B and P-glycoprotein with increase of adverse effect (Van den Bout-Van den Beukel et al., 2006);
- ✓ Phosphodiesterases-5 inhibitors such as sildenafil. The juice inhibits CYP3A4, CYP1A2, and P-glycoprotein and increased bioavailability. The effect includes increase of adverse effect (Bailey & Dresser, 2004). Irreversible inactivation of intestinal CYP3A4 was produced by commercial grapefruit juice given as a single normal amount (200-300 mL) or by whole fresh fruit segments. Enhanced oral drug bioavailability can occur 24 hours after juice consumption;
- ✓ Antihistamines, such as terfenadine, fexofenadine. The juice inhibited CYP3A4, CYP1A2, MRP2, OATP-B and P-glycoprotein with increase of adverse effect (Dresser et al., 2005). Grapefruit juice at the doses of 300 mL and 1200 mL was ingested concomitantly with 120 mg fexofenadine by 12 healthy volunteers in a randomized 4-way crossover study, and fexofenadine pharmacokinetics were determined over a period of 8 hours. The 300 mL of grapefruit juice decreased the mean area under the plasma drug concentration-time curve and the peak plasma drug concentration of fexofenadine to 58% and 53%, respectively, while 1200 mL grapefruit juice reduced these parameters to 36 and 33%, respectively.

- ✓ Antiarrhythmics such as amiodarone. To eleven healthy adult volunteers, was given a single oral dose of amiodarone (17 mg/kg) and 300 mL of grapefruit juice in the same day. The juices inhibited of CYP3A4, CYP1A2, MRP2, OATP-B and P-glycoprotein and increased bioavailability was observed. The juice increases the adverse effect of drug (Libersa et al., 2000), such as torsade de pointes, a rare but sometimes fatal ventricular arrhythmia, which occurs in the context of QT interval prolongation (Saito et al., 2005);
- ✓ Antibiotics such as erythromycin. The effects of grapefruit juice intake on the pharmacokinetics of erythromycin were investigated in 6 healthy male volunteers, who received 400 mg erythromycin with grapefruit juice. The juice inhibits CYP3A4, CYP1A2, MRP2, OATP-B and P-glycoprotein and increased bioavailability with consequent increase of adverse effect (Amory & Amory, 2005). In this study, administration of grapefruit juice with erythromycin in humans significantly increases the resulting maximal plasma concentration of erythromycin (by 52 %) and the area under the curve (by 49%). More prolonged administration results in even greater inhibition of CYP3A4, increasing plasma levels of erythromycin further and potentially increasing the risk of sudden death from QT-interval prolongation. It has been proposed that a psoralen compound, 6'7'-dihydroxybergamottin, which is found in grapefruit juice but not in orange juice, is responsible for this inhibition;
- ✓ Oral contraceptives such as ethinylestradiol. The juice inhibited its metabolism and enhanced risk of deep vein thrombosis (Yadav et al., 2020);
- ✓ Opioids such as oxycodone or fentanyl. The juice inhibited the CYP3A4 and enhance respiratory depression risk (Yadav et al., 2020).
- ✓ Benzodiazapines such as Ddazepam, midazolam triazolam. Eight healthy subjects participated in this study received an oral dose of diazepam (5 mg) with 250 mL of grapefruit juice. The juice inhibited benzodiazapine metabolism and caused excessive sedation and amnesia (Ozdemir et al., 1998).

Thus, it is best to avoid oral co-administration of orange juice with these drugs to ensure therapeutic efficacy and potency (Yadav et al., 2020).

Recurrent intake of grapefruit juice determined a selective reduction of both CYP3A4 and CYP3A5 protein expression in enterocytes, with increase of drug bioavailability (Lown et al., 1997). Messenger RNA expression is not reduced, suggesting that this reduction in activity is not transcriptionally mediated. The mechanism of the reduction in CYP3A4 protein most likely reflects either accelerated protein degradation or reduced messenger RNA translation. It would be rational to suppose that one or more constituents of grapefruit juice determined a rapid intracellular degradation of the intestinal CYP3A4 enzyme through irreversible "suicide" inhibition that explain the rapid and sustained onset of inhibition by grapefruit juice (Schmiedlin-Ren et al., 1997). In intestinal, a 47% reduction of CYP3A4 concentration was observed within 4 hours of the ingestion of grapefruit juice, and grapefruit juice maintained a bioavailability-enhancing effect for up to 24 hours (Lundahl et al., 1995).

In a randomized three-way crossover design, 10 volunteers received a felodipine 10 mg extended-release tablet with 240 mL of Seville orange juice. Orange juice not altered CYP3A4 activity but the pharmacokinetic parameters of CYP3A4 substrates (Malhotra et al., 2011). In addition, the authors reported that after Sevilla orange juice intake, increase of 76% in felodipine bioavailability was observed. The effect was attributable to a significant concentration of bergamottin and 6,7-dihydroxybergamottin in orange juice that exerts inhibitory effects on Pgp-mediated drug efflux.

The tangerine juice (a kind of Mandarin orange), decreased the AUC of midazolam by about 40% over the first 1.5 hours and increased Tmax 2-fold without effects on total AUC, Cmax and the AUC ratio of the main metabolite to midazolam (Saito et al., 2005). Tangerine juice might have some impact on the absorption process of midazolam.

Lime juice demonstrated mechanism-based inhibition of CYP3A4 activity *in vitro*. However, in a clinical study, 25% diluted lime juice containing the candidate causative ingredient bergamottin in the same amounts as grapefruit juice did not exert any significant effects on a pharmacokinetic parameter of felodipine (Saito et al., 2005).

Many compounds, both flavanoids and non-flavanoids, have been offered as the active inhibitory ingredients in grapefruit juice. Grapefruit contains several flavonoids (mainly as glycosides), which are presumed to be electron-rich substrates for CYP450 enzymes that are ultimately hydrolysed to the corresponding aglycons and sugar by intestinal microflora. Naringin, the glycoside of naringenin, is found in grapefruit juice in concentrations of 450 μg/mL (10% of the dry weight of juice), making it the most abundant flavanoid in grapefruit juice (Kane & Lipsky, 2000). Naringin has no effect on the activity of the human CYP system *in vitro*, but its metabolite naringenin is a potent inhibitor of both the CYP3A and CYP1A2 isoforms *in vitro*. However, *in vivo* oral naringenin only weakly inhibits CYP3A4 and CYP1A2. The inability of oral naringin to markedly inhibit intestinal CYP3A4 likely implies that it is not the main active agent in grapefruit juice. Studies of other flavonoids have yielded similar results, inhibition in the Petri dish but not in the intestine (Edwards & Bernier, 1996).

In three studies, naringin, given as an ‘aqueous solution’, or in an ‘encapsulated’ preparation, in the same amounts as present in grapefruit juice had no effect on nitrendipine, felodipine or nisoldipine pharmacokinetics (Kane & Lipsky, 2000).

In parallel to beneficial proprieties, coumarins have controversial effects on humans, due their ability to interact with several drugs. The controversial effects of furanocoumarins are related to the ability to inhibit the intestinal CYP3A4 through inhibition of basic mechanisms. For patients undergoing drug therapy, the inhibition of cytochrome P450 by furanocoumarins may lead to a higher concentration of drug in the blood, which in turn can cause serious side effects such as heart rhythm disturbances or respiratory depression (Dugrand-Judek et al., 2015). This effect is often referred to as "the effect of grapefruit juice". Masuda et al. (2018) reported the capacity of grapefruit juice to increase the oral availability of nifedipine, cyclosporin A, midazolam, triazolam, and tacrolimus. The CYP3A4 inhibition was mainly attributed to bergaptol and its derivatives such as bergaptene, isoimperatorin, and bergamottin.

Many of these constituents of grapefruit juice are present as a mixture of chiral isomers that vary markedly in proportion and concentration, depending on the maturity of the fruit and the method of juice extraction and purification. It is possible that the inhibition of first-phase intestinal metabolism by grapefruit juice is mediated by a combination of flavonoid and furanocoumarin compounds and not singly (Kane & Lipsky, 2000).

2.2 *Citrus* × *clementina*

2.2.1 Botany and chemistry

Citrus × *clementina* Hort. ex Tan. (Figure 2) is one of the most important crop variety of *Citrus* mandarins in the Mediterranean basin. It is a hybrid between mandarin and orange discovered by Father Clemente Rodier in Algeria in the early 20th century and cultivation spread between 1930 and 1950, where this *Citrus* fruit found its ideal habitat (Benabdelkamel et al., 2012). Due to their sweet juicy pulp and absence of seeds, these fruits are recognized all over the world. The fruits are roundish, with epicarp of dark orange colour and smooth, rich in oil glands.

Fig. 2. *Citrus* × *clementina* fruit

The clementine tree is very similar to that of mandarin, from which it differs slightly for the leaves that are larger. It blooms and fructifies slowly and irregularly, as it is very susceptible to temperature changes. There is only one annual harvest between November and January. Clementine grow on different continents, but Italy is among the major European producers. In particular, in Calabria, south of Italy, the cultivation of clementine is widespread for to optimal climatic conditions that have contributed to development of food product awarded with protected geographical indications (PGI) certification by the European Union as “Clementine di Calabria” (EC, 1997).

Economically frauds are surveyed because fruits produced in other countries, such as Spain, Tunisia, and Algeria, are often introduced in the market with the Calabria PGI brand name. The Consortium for the protection of the PGI “Clementine of Calabria” was therefore founded in 1998 to promote and protect the autochthon products according to the standard UNI 22005:2008.

In order to evaluate the implication of *Citrus* flavanones on human health, it is necessary to consider the differences in their metabolism and absorption in body. The ability of *Citrus* flavanones to exert beneficial effects depends on their bioavailability, correlated by the structure of the compound, the food matrix, and host factors. The

intestinal metabolism of these compounds is determined by their degree of conjugation to sugar moieties and the removal these by intestinal bacteria (Stevens et al., 2019).

Hesperidin and naringin represents the main flavanone glycosides know in *Citrus* fruits. These compounds, after intake, are resistant to enzymatic breakdown in the stomach and small intestine and, therewith, mainly reach the colon intact (Stevens et al., 2019). Hesperidin and naringin, containing rutinose groups (glucose and rhamnose sugars at position 7), are hydrolysed only in the distal part of the intestine and the colon by the gut microbiota bacteria, α-rhamnosidases, that remove rhamnose moiety, followed by the removal of glucose by β-glucosidases. In contrast, the flavonoid glucosides, containing only a glucose moiety, are hydrolysed already in the small intestine by β-glucosidases.

Consequently, the aglycones hesperitin and naringenin are released, absorbed through the intestinal epithelium by means of passive diffusion and proton-coupled active transport, transformed by human cell enzymes into phase II conjugates including, glucuronides and sulfates, resulting in their glucuronidated and sulfated derivatives, and distributed at the various cell sites. Hesperetin-7-*O*-β-D-glucuronide, hesperetin-3'-*O*-β-D-glucuronide, hesperetin sulfoglucuronide, and hesperetin diglucuronide are the main metabolites of hesperetin (Matsumoto et al. 2004; Mullen et al. 2008). While, naringenin-7-*O*-β-D-glucuronide, naringenin-4'-*O*-β-D-glucuronide, naringenin-4'-*O*-sulfate-7-O-β-D-glucuronide, naringenin-4',7-*O*-disulfate, and naringenin-4'-*O*-β-D-glucuronide-7-*O*-sulfate are the most abundant metabolites of naringenin (Brett et al. 2009; Tripoli et al. 2007). Through enterohepatic recirculation, these conjugated compounds are excreted by the liver as components of bile into the intestine. Additionally, the deconjugated compounds are regenerated by microbial enzymes before being reabsorbed (Tomas-Navarro et al., 2014). The aglycones are further metabolized into phenolic acids and simple phenolics by C-ring cleavage, demethylation and dihydroxylation by bacterial enzymes (Stevens et al., 2019). In clinical studies exist relevant factors should be considered when assessing the biological effects after flavonoids intake (Tomas-Navarro et al., 2014):

a) difference in gut microbiota that affect the deconjugation of flavanone rutinosides in the gut and therefore their absorption. Colon microbiota is responsible for this hydrolysis, and species of the genera Bacteriodes and Eubacterium have been suggested to be responsible for this glycosidase activity. The absorbed aglycones are metabolized by phase II enzymes to yield the corresponding conjugates, and they circulate in plasma and reach urine in these conjugate forms. One of the potential explanations of the differences found in absorption is that the native glycosides are not hydrolysed to aglycones;
b) difference in the expression of phase II enzymes and transporters in the intestinal epithelial cells. Extensive research using cell and animal models has identified a number of enzymes and transporters in the human gut epithelial cells that interact with flavonoids and flavonoid glycosides and play a role in their uptake, metabolism and transport. These include β-glucosidases, the apical Na-dependent

GLUT1, apical and basolateral multi-drug resistance protein (MRP) transporters, UDP-glucuronosyl transferase and sulfotransferase conjugating enzymes;

c) difference in the physical form in which flavanones are ingested such as juice, soluble extract, capsule. Increasing concentrations of the flavanones in the juice affect the proportion of soluble/insoluble compounds in the haze and, therefore, have an effect on the absorption and metabolism of these compounds. The type of beverage (hands queezed, processed) and the ratio 'time-temperature' of storage also have an effect on flavanone absorption. The insoluble fraction inhibits absorption, while the increase flavanone solubility in beverages leads to an increase in their bioavailability. Not only the flavanone content but also the solubility of the flavanones was relevant in terms of flavanone absorption in beverages;

Naringenin and its glucuronides were found in plasma and brain of rats 10 min after administration of a concentration of 20 mg/kg (Peng et al. 1998), while, higher concentrations of this compound were detected in the bile and liver (Tsai 2002). Erlund et al. (2001) reported the concentration in human plasma of naringenin and hesperetin aglycones after ingestion of grapefruit and orange juice (8 mL/kg bw). A range of 0.6–6.0 mmol/L with a peak concentration of 6.0 mmol/L for naringenin from grapefruit juice and 2.2 mmol/L for hesperetin from orange juice was found. Additionally, the authors suggested a considerable distribution to tissues for both flavanones. After ingestion of 1 L of orange juice (characterized by a content of 444 mg of hesperidin and 96.4 mg of narirutin), analysis over 24 h led to Cmax values at 1.28 and 0.20 mmol/L for hesperetin and naringenin (after deconjugation), respectively. The levels in urine of flavanones, expressed as percentage of their intake, amounted to 7.9% and 6.4% for naringenin and hesperetin, respectively. The dose not considerably affected the urinary excretion of flavanones (Manach et al. 2003).

Generally, naringenin is more bioavailable than hesperetin as resulted by both plasma and urine analyses (Gardana et al. 2007; Kanaze et al. 2007). Successively, the ingestion of 135 mg of both hesperetin and naringenin, under fasting conditions, in 6 volunteers resulted in their appearance as metabolites in blood plasma 20 min later. Peak plasma concentration of 2.7 and 7.4 mM for hesperetin and naringenin was reached 4.0 and 3.5 h after ingestion, respectively (Kanaze et al. 2007).

Polyphenols

Polyphenols comprise a variety of bioactive compounds, commonly divided into several classes including flavonoids, hydroxycinnamic acids, anthocyanins, proanthocyanidins, stilbenes and lignans (Cirmi, 2016).

Flavonoids

The basic structural feature of flavonoid is 2-phenyl-benzo-γ-pyrane nucleus, contains a C6-C3-C6 heterocyclic skeleton, consisting of two benzene rings linked through a heterocyclic pyran ring (Figure 3). Based on the oxidization of the heterocyclic (C3) ring,

Citrus flavonoids can be divided in flavanones, flavonols, and flavones. Anthocyanins are considered as metabolites of flavones (Cirmi et al., 2016)

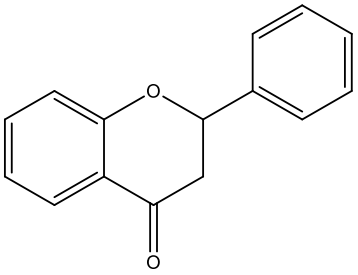
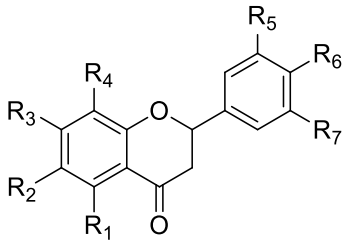


Fig. 3. Flavonoids structure

Flavanones includes approximately 95% of the total flavonoids and their content depends on the age of the plant. In *C.* × *clementina*, the most important flavanones present in the aglycone forms are naringenin and hesperetin (Figure 4).

Among flavanones with neohesperidose, naringin, neoeriocitrin, neohesperedin, and poncirin are the most abundant. Hesperidin, narirutin, eriocitrin, and didymin are the main flavanones with rutinose (Loizzo et al., 2018).



NAME	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Naringenin	OH	H	OH	H	H	OH	H
Hesperetin	OH	H	OH	H	OH	OCH_3	H
Narirutin	OH	H	O-Rut	H	H	OH	H
Naringin	OH	H	O-Neo	H	H	OH	H
Poncirin	OH	H	O-Neo	H	H	OCH_3	H
Eriocitrin	OH	H	O-Rut	H	OH	OH	H
Neoeriocitrin	OH	H	O-Neo	H	OH	OH	H
Hesperidin	OH	H	O-Rut	H	OH	OCH_3	H
Neoesperidin	OH	H	O-Neo	H	OH	OCH_3	H
Dydymin	OH	H	O-Rut	H	H	OCH_3	H

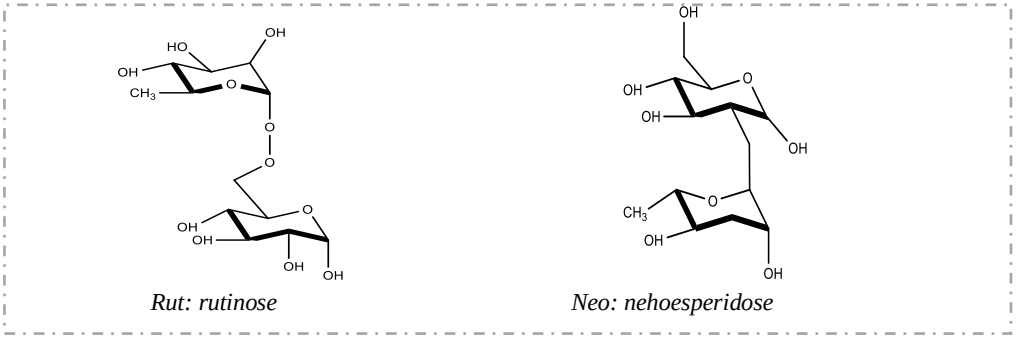


Fig. 4. Flavanones structure

Citrus flavanones are recognized for their beneficial properties on human health. Great attention was focused on hesperidin and its aglycone form, hesperetin, which play an important role in the prevention of diseases associated with oxidative stress such as obesity, diabetes, inflammation and cancer (Barreca et al., 2017). The treatment of human

neuroblastoma SK-N-SH cells with hesperidin reduced ROS generation and exerted neuroprotective effects in a dose-dependent manner (Tamilselvam et al., 2013).

Hesperidin antioxidant mechanism of was correlated to direct ROS scavenging, transition metal ion chelation and its ability to increase cellular glutathione content.

Recently, De Souza et al. (2016) compared the antioxidant activity of hesperidin, hesperetin and G-hesperidin *in vitro* and *in vivo*, administrating each of these for 30 days at 1 mmol/kg body mass (bm) to Wistar male rats. The data showed that the aglycone form has the greatest inhibitory activity of xanthine oxidase by increasing superoxide dismutase activity in the liver of animals. The administration 500 mg for three weeks of hesperidin in patients with metabolic syndrome reduced total cholesterol and apolipoprotein B (APO B) levels, while high density lipoprotein (HDL) level was increased. A dose of 100 mg or 500 mg for six weeks in subjects with hypercholesterolemia, the hesperidin decreased serum triglycerides and low-density lipoprotein (LDL) levels (Li & Schluesener, 2017).

In addition, intra-gastric hesperidin attenuates the increased level of plasma cholesterol, low density of lipoprotein-cholesterol, very low-density lipoprotein-cholesterol, triglycerides, free fatty acids and phospholipids and decreased level of high-density lipoprotein-cholesterol (Homayouni et al., 2017). The metabolic effects of hesperidin were demonstrated by Sahnoun et al. (2017) and Zeng et al. (2018) that reported its ability to inhibit α-amylase, α-glucosidase and lipase enzyme with IC_{50} value of 111 and 1 μM, and 688,25 μg/mL, respectively.

In a randomized double-blind controlled clinical trial design, 23 subjects with T2DM consumed 500 mg/day hesperidin supplement and 22 subjects were treated with placebo (control group) for 8 weeks. Hesperidin supplementation led to significant decrease in fasting blood glucose and glycated haemoglobin. A significant increase in serum insulin and decrease in triglyceride were also observed in the hesperidin treated group (Eghtesadi et al., 2016). Similarly, Mohammadi et al. (2016) reported that supplementation with hesperidin (500 mg/day for 8 weeks) in T2DM patients (n= 45) resulted in a reduction in fasting blood glucose, total cholesterol and hemoglobin A1C at the same time a significant increase in serum insulin was detected.

The anti-inflammatory activity of hesperidin in various animal models and cell types were recently demonstrated. Hesperidin, in fact cause a reduction of pro-inflammatory cytokines such as, IL-1β and IL-6, and TNF-α (Yamamoto et al., 2013). In addition, the treatment with 500 mg hesperidin significantly reduced the plasma levels of C-reactive protein and serum amyloid A in individuals with metabolic syndrome (Homayouni et al., 2018).

The cardioprotective effect of hesperidin was investigated by Haidari et al. (2015). Administration of 600 mg/day of hesperidin decreases levels of adiponectin and HDL-C and increases E-selectin in patients with myocardial infarction. Previously, the protective effect of hesperidin was investigated by Kakadiya et al. (2010) that reported its capacity to reduce systolic and diastolic blood pressure in diabetic rats.

Morand et al. (2011) investigated the effect of orange juice and its major flavonoid hesperidin on microvascular reactivity, blood pressure, and cardiovascular risk biomarkers through both postprandial and chronic intervention studies in twenty-four healthy overweight men. Diastolic blood pressure was significantly lower after 4 weeks consumption of orange juice (500 mL) or control drink+hesperidin (500 mL) compared to placebo.

The neuroprotective activity of hesperidin was evaluated by Thenmozhi et al. (2015). In this study, the administration of 100 mg/kg of hesperidin along with aluminium chloride ($AlCl_3$) injection for 60 days, significantly reduced the concentration of ROS in hippocampus and cortex, the AchE activity, the protein expressions of amyloid precursor protein, the levels of both $A\beta_{1-42}$ and β and γ secretases. Administration of 200 mg/kg significantly attenuated behavioural, biochemical, and mitochondrial alterations probably due a mechanism of action that involves NO–cyclic guanosine monophosphate (cGMP) pathway (Kumar et al., 2013). More recently, Li & Schluesener (2017) demonstrated that administration of100 μg/kg of hesperidin for 10 days significantly attenuated β-amyloid deposition and microglial activation in brain of transgenic mice.

López Muñozmaría et al. (2015) reported that combination of diosmin and hesperidin exerted analgesic and/or anti- inflammatory effects (Patent no. WO2015019334). This application was used for the treatment of pain: moderate to severe pain, chronic pain, and/or neuropathic pain. No occurrence of adverse effects was observed. Indeed, Li et al., (2019) reported that hesperidin has median lethal dose (LD_{50}) of 4837.5 mg/kg, and Low Observed Adverse Effect Level (LOAEL) at 1000 mg/kg for both male and female Sprague-Dawley rats.

Hesperetin represents the major aglycone metabolite of hesperidin and displays several biological properties (Cirmi et al., 2016). Jayaraman et al. (2018) investigated the anti-hyperglycaemic, antioxidant and anti-hyperlipidaemic effects of hesperetin against streptozotocin (STZ)-induced experimental rats. Supplementation with 40 mg/kg of hesperetin for 45 days determined a significant decline in plasma glucose level and a marked improvement in insulin and glycogen secretion.

Previously, Rizza et al. (2011) reported that dietary supplementation of 500 mg hesperetin for three weeks on adults affected by metabolic syndrome induced an improvement in brachial artery flow mediated by dilation and a decrease in inflammation markers, such as C-reactive protein and serum amyloid A protein.

Hesperetin, is also known for its capacity to induce apoptosis in cancer cells primarily through activation of caspase-9 (Farooqi et al., 2015). Together with an increase of intracellular β-catenin, mitogen-activated protein kinases, and cyclic adenosine monophosphate (cAMP)-responsive element binding protein level (Huang et al., 2012). Recently, Samie et al. (2018) investigated on capacity of hesperitin to attenuate testicular alteration in Wistar rat trough inhibition of inflammation, oxidative stress, and apoptosis. Li et al. (2018) reported the ability of hesperetin, to inhibit the chloride channel and propose its use for the treatment of diarrhoea, heart disease, pulmonary disease, stomach,

brain and mental disease, rhinitis, ontological disease and eye disease drug development (Patent no. CN108815154A).

Hesperetin administered orally at the doses of 50 mg/kg/day for 46 days reduced reactive oxygen species, DNA fragmentation, serum glucose, MDA levels and caspase 3 activity. In addition, hesperetin potentiated testicular antioxidant system with consequent increase of glutathione levels, ferric reducing antioxidant power, catalase (CAT), SOD, and glutathione peroxidase (GPx) activity in diabetic rats (Samie et al., 2018).

The neuroprotective properties of hesperetin was recently demonstrated by Shagirtha et al. (2017). The oral administration of this flavanone (40 mg/kg bw for 21 days) protected the brain of Wistar rats by increasing the levels of enzymatic antioxidants such as CAT, SOD, GPx and glutathione S-transferase (GSTs). Tomas-Navarro et al. (2014) compared the antioxidant activity of hesperetin and neohesperidin. The results showed that hesperetin had a greater antioxidant power *in vitro* than neohesperidin, which structurally are characterized by introduction of neohesperidose that influences mechanisms of the antioxidant activity.

Jia et al. (2015) reported the *in vivo* hypoglycaemics and hypolipidemic effects of neohesperidin on KK-A(y) mice. Treatment with neohesperidin significantly decreased serum glucose, fasting glucose, glycosylated serum protein, and decreased insulin resistance. Moreover, this bioactive compound significantly decreased total cholesterol, serum triglycerides, leptin level, and inhibited lipid accumulation. In addition, Lv et al. (2015) noted that naringin and neohesperidin mainly inhibited amylose digestion.

Shi & Yang (2018) discloses a novel pharmaceutical use of neohesperidin in the preparation of a drug for treating bronchial asthma, or diseases caused by Th1/Th2 cell immune imbalance (Patent no. CN108478586). Oral acute toxicity of hesperetin is >2000 mg/kg bw (Vaeth, 2006).

The efficacy of *Citrus* flavonoids on metabolic syndrome resulted into the commercialization of Bergavit® (Bionap, Italy), a standardized extract containing 150 mg of the main active flavonoids of bergamot juice (16% of neoeriocitrin, 47% of neohesperidin, and 37% of naringin). This supplement was administrated at a fixed dose daily for 6 months in patients with moderate hypercholesterolemia. Results revealed a triglyceride, total cholesterol, and LDL-cholesterol reduction (Toth et al., 2016).

Previously, Mollace et al. (2011) tested the ability of neoeriocitrin, naringin, neohesperidin to reduce the cholesterol, LDL-C, triglycerides and glucose levels but also the increase HDL-C levels in 237 patients suffering of hyperlipidaemia either associated or not with hyperglycaemia.

Naringin, as reported by Sahnoun et al. (2017), showed an excellent inhibition for α-amylase and α-glucosidase enzyme, with IC_{50} values of 8.0 and 0.55 μM, respectively.

Lim et al. (2018) studied the protective effects and molecular mechanisms of naringin in diabetic mice. The results showed that this flavanone dose-dependently ameliorated hyperglycaemia and protected STZ-induced β-cell death by inhibiting both the intrinsic and extrinsic apoptotic pathways. These protective effects have been related to the ability of naringin to reduce ROS and pro-inflammatory cytokines accumulation. These results

agree with those reported by Mahmoud et al. (2012). Naringin, in fact, could decrease the levels of glycosylated haemoglobin, glucose, MDA, TNF-α, IL-6, NO and increase the levels of glutathione, serum insulin, vitamin E, vitamin C. Alam et al. (2013) reported that supplementation with naringin (100 mg/kg/d) in high-fat/high-carbohydrate-diet–fed obese rats decreased cholesterol and triglycerides levels, reduced abdominal fat deposition and ameliorated the endothelial dysfunction.

Similar effects were observed also by Park et al. (2013) in C57BL/6 mice in which naringin supplementation (0.02%) decrease plasma and liver cholesterol concentrations and ameliorated indices of insulin sensitivity. In addition, dietary supplementation with naringenin (0.003, 0.006 and 0.012% for 6 week) or naringin (25-100 mg/kg for 28 day) induced a decrease of plasma and hepatic triglycerides and cholesterol and improved insulin sensitivity (Alam, 2014). In a clinical study, administration of naringin (400 mg/capsule/day) for 8 weeks in hypercholesterolemic individuals resulted in reduced plasma total cholesterol and LDL cholesterol concentrations. Additionally, erythrocyte SOD and CAT activities were significantly increased, while GSH-Px activity and plasma TBARS levels were reduce after naringenin treatment (Jung et al., 2003). This compound was able, also, to exert its antidiabetic effect by the inhibition of gluconeogenesis and upregulations of AMPK hence metformin-like effects. In addition, it increases glucose uptake in skeletal muscles, ameliorated pro-inflammatory reactions, prevented metabolic dysregulation and atherosclerosis (Nyane et al., 2017).

The *in vivo* intestinal α-glucosidase inhibitory activity induced by naringin was also demonstrated by Priscilla et al. (2014). Moreover, naringenin decreased blood glucose, serum lipid, and ameliorated glucose tolerance through down-regulating oxidative stress and inflammation in STZ-induced rats (Jia et al., 2015). Administration of a *Citrus* polyphenol extract (Sinetrol-XPur, 900 mg/kg) (Sinetrol®), containing naringin for 12 weeks in overweight individuals, resulted in significantly reduced body weight and plasma glucose levels compared to the control placebo overweight group (Dallas et al., 2014). Liang et al. (2015) reported the protective effect of naringenin and naringin in tumour radiotherapy (Patent no. CN104940932A). The use of naringenin, naringin and salts in preparation of pharmaceutical compositions for the treatment of cough and phlegmatically conditions, was assessed by Wei-Wei et al. (2004). These compounds determined improve of cough and phlegmatically diseases, without side effects, and can be used to treat acute and chronic bronchitis and cold (Patent no. WO2004064848A1). Additionally, Liao (2018) reported the use of naringenin and its derivative in preventing AD and or other cognitive disorder diseases (Patent no. CN108785301A).

The anti-inflammatory activity of naringenin was confirmed by Wu et al. (2016) that showed how this compound inhibited the expression of cytokine signalling, iNOS, COX-2, release of NO and pro-inflammatory cytokines in microglial cells. A direct effect of this flavanone determined down-regulation of genes involved in *de novo* lipogenesis, lipolysis and triglyceride synthesis/storage. Another possible target of obesity treatment consists in the pancreatic lipase inhibition. Zeng et al. (2018) demonstrated that narirutin and didymin are able to inhibit this enzyme with IC_{50} values of 58.98 and 67.30 μg/mL,

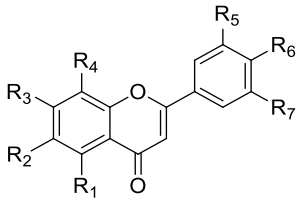
respectively. Naringenin rat oral toxicity was reported by Radhakrishnan et al. (2018) that found LD_{50} value of 5520 mg/ kg bw.

More recently, Ali et al. (2019 b) demonstrated that didymin was able, also, to inhibit of α-glucosidase, α-amylase enzymes and increase glucose uptake. In addition, didymin reduced the expression of two key enzymes involved in the gluconeogenesis such as glucose 6-phosphatase and phosphoenolpyruvate carboxykinase with a consequent decrease of glucose production. The anti-obesity effect was proved also for poncirin that showed IC_{50} values of 125.5 and 8.0 μM against α-amylase and α-glucosidase, respectively (Tundis et al., 2016). Additionally, Bi (2012) reported the application of didymin in preparation of drugs for treatment or prevention of liver diseases, such as hepatic fibrosis (Patent no. CN103845351A).

Eriocitrin, is known as a strong antioxidant agent (Smeriglio et al., 2019). It has been shown that a major role is played by two hydroxy groups that in this flavonoid are bound to the B ring in ortho position with respect to each other (Diab et al., 2015).
This flavanone at dose of 200 mg/kg showed protective effects against inflammation and oxidative stress in C57BL/6J mice, and may therefore prevent metabolic alterations associated with the development of cardiovascular diseases (Ferreira et al., 2016).
More recently, Eun-Young & Myung-Sook, (2020) proposed a novel mechanism of eritrocin action in improving obesity and related metabolic disorders such as hyperlipidemia, and insulin resistance. Dietary supplementation with eriocitrin (HFD + 0.005%, w/w) in C57BL/6N mice for 16 weeks, improved adiposity by increasing adipocyte fatty acid oxidation, energy expenditure, mRNA expression of thermogenesis-related genes in in brown adipose tissue and skeletal muscle, and by decreasing the expression of lipogenesis-related genes in white adipose tissue. The supplementation also decreased hepatic lipogenesis and prevented hyperlipidaemia, whereas it increased hepatic FA oxidation and fecal lipid excretion, thus suggesting amelioration of HFD-induced hepatic steatosis. Moreover, eritrocin supplementation improved insulin resistance, glucose tolerance, and it decreased hepatic gluconeogenesis and pro-inflammatory responses. Additionally, as reported by Liu et al. (2019) eriocitrin has a potential to become a novel diabetes treatment medicine for suppressing activity of α-glucosidase (Patent no. CN109806272A). Radhakrishnan et al. (2018) after eriocitrin oral administration reported a DL_{50} value of 6750 mg/Kg bw.

In *Citrus* fruits, flavone and its glycosides (Figure 5) represented the second major group of flavonoids. These compounds differ from flavanone due the presence of flavones of a double bond in positions C-2 and C-3. The most commonly flavones found in C. × *clementina* fruits and by-products are apigenin, luteolin and diosmetin (Loizzo et al., 2018). For flavone glycosides, the most common sugar moieties include glucose, rutinose, and neohesperidose (Silberberg et al., 2006). Both the C-6 and C-8 positions can be C-glycosylated, whereas the C-7 position is the most favourable for O-glycosides.

Nobiletin is one of the most abundant polymethoxylated flavones. This compound was investigated for their capacity to improve and prevent obesity and metabolic disease.



NAME	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Apigenin	OH	H	OH	H	H	OH	H
Luteolin	OH	H	OH	H	OH	OH	H
Sinensetin	OCH_3	OCH_3	OCH_3	H	OCH_3	OCH_3	H
Tangeretin	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H
Nobiletin	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H

Fig. 5. Flavones structure

More recently, Li (2019) reported the application of nobiletin in preparation treatment gastric accommodation disorder remedies. This compound selectively relaxes stomach smooth muscle and promote the recovery of physiological gastrointestinal motility, and calms stomach upset. As new therapeutic agent, provides and presented great market prospects and economic value (Patent no. CN108619130B).

Sahnoun et al. (2017) reported the carbohydrate hydrolysing enzymes inhibitory activity of nobiletin with IC_{50} value of 42.0 and 50.0 μM against α-amylase and α-glucosidase, respectively. This flavone was able, also to inhibit lipase with IC_{50} value of 26.28 μg/mL (Zeng et al., 2018). It is noteworthy that this IC_{50} value is better that those reported for the positive control. In db/db diabetic mice orally administration of nobiletin at dose of 200 mg/kg bw for10-week significantly attenuated body weight gain, decrease fasting glucose levels, improved glucose tolerance and insulin sensitivity, and diminished serum triglycerides levels (He et al., 2016). Moreover, Lone et al. (2018) demonstrated that nobiletin was able to reduce the protein peroxisomal acyl-coenzyme A oxidase 1, carnitine palmitoyltransferase-1 and ameliorated fatty acids β-oxidation *via* AMP-activated protein kinase activation. In addition, treatment with this compound at 10-100 mg/kg for 8 weeks in obese mice accelerated lipid catabolism in adipose tissue.

Previously, the blood lipid lowing effects of nobiletin in liver hepatocellular cells (HepG2) was observed. This flavone inhibited apolipoprotein B secretion, triglyceride and cholesterol synthesis with IC_{50} values of 29, 73 and 68 μM, respectively (Lin et al., 2011). Intracellular lipid accumulation was significantly reduced in 3T3L1 adipocytes after treatment with nobiletin at the dose of 64 μM, while in C57BL6/J mice, supplementation with 100 mg/kg diminished adipose tissue expression of both CCL2 and IL-6 chemokine (Lee et al., 2013).

Moreover, nobiletin inhibits the generation of NO, prostaglandin E2, TNF-α, IL-1β and IL-6 (Patent no. KR100770746B1) and prevent inflammatory diseases Se-jae et al. (2006). The anti-inflammatory capacity of nobiletin, *via* down-regulation of COX-2 and iNOS expression, was evaluated by Xiong et al. (2015). In hepatocytes, nobiletin suppresses the induction of NO with an IC_{50} value of 50 μM and decreased iNOS expression (Yoshigai et al., 2013). The neuroprotective role of nobiletin was reported by Nakajima et al. (2014). In this study nobiletin, administered at dose 30 mg/kg for 3

months, reversed the impairment of short-term memory and recognition memory in a triple transgenic mouse model of Alzheimer's disease (AD).

More recently, Qi et al. (2019) demonstrated that orally administration of nobiletin (100 mg/kg/day for 6 weeks), ameliorated LPS-triggered memory deficit regarding synaptic dysfunctions and neuronal loss, inhibited the microglial activation and pro-inflammatory cytokine secretion (IL-1β, COX-2, TNF-α, and iNOS). In addition, in BV-2 microglia cells, the action of this flavone decrease pro-inflammatory cytokines secretion trough modulation of mitogen-activated protein kinase (MAPKs), phosphatidylinositol 3-kinase/phosphorylated protein kinase B (PI3K/Akt), and NF-κB signalling pathways.

Ma et al. (2014) investigated the inhibitory effect of nobiletin in SMMC-7721 hepatic cancer cells as well as in H22 transplantable tumor. The mechanism of antiproliferative action involves down-regulation of COX-2expression, up-regulation of Bax and caspase-3 expressions. Additionally, Chen et al. (2014) reported that nobiletin reduce human ovarian cancer cells viability and human breast cancer cells through the reduction of key mediators in angiogenesis, such as Akt, hypoxia-inducible factor 1-α (HIF-1α), vascular endothelial growth factor (VEGF), NF-κB. Several potential nobiletin health benefit should be related to its antioxidant proprieties. After nobiletin oral administration in mouse, Xu & Wang (2020), reported a DL_{50} value of 780 mg/kg bw. Additionally, Wen-zhe et al. (2015) detected a pharmaceutical composition for multidrug resistance cancer treatment, comprising a *Citrus* methoxyflavone (nobiletin) and a chemotherapeutic drug (Patent no. US9808477B2). Chen & Wang (2015) proposed application of nobiletin in preparation of health products or medicines for prevention and/or treatment of oral cancer (Patent no. CN105030559A). Through the anti-proliferation effects of hesperetin, naringenin and nobiletin on human oral epidermoid carcinoma cells, the experiments showed that these compounds possessed an obvious effect on inhibiting proliferation of human oral epidermoid carcinoma cells.

Both nobiletin and tangeretin, ameliorated ROS production and lipid peroxidation in mutant *Saccharomyces cerevisiae* deficient in glutathione synthase, SOD, or CAT (Wang et al., 2018). Similarly, Liu et al. (2019) observed a significant decrease in ROS content, with increase in the activities of SOD, CAT, and GPx through inhibition of NF-κB pathway in rat insulinoma cell line (INS-1) pre-treated with tangeretin (0, 10 or 20 μM) for 12 hours.

Oxidative stress plays an important role in development of disease, such as diabetes mellitus, obesity and cancer. Recent report elucidated the anti-obesity capacity of tangeretin *via* inhibition of pancreatic lipase. This compound in fact inhibited the enzyme with IC_{50} value of 57.31 μg/mL (Zeng et al., 2018). Moreover, tangeretin ameliorated insulin resistance and increased glucose uptake by attenuating obesity-induced inflammation in adipose tissue through reduction of NO production, the expression of IL-6, IL-1β, TNF-α, iNOS, and COX-2 in a co-culture of 3T3-L1 adipocytes and macrophage cell line (RAW 264.7) (Shin et al., 2017). Sahnoun et al. (2017) evaluated the inhibitory activities of tangeretin on carbohydrate metabolism key enzymes. This pentamethoxy flavone showed an IC_{50} value of 141.0 and 14.8 μM against α-amilase and α-glucosidase,

respectively. Previously, Sundaram et al. (2014) reported that oral administration of tangeretin (100mg/kg bw for 30 days) to diabetic rats decreased levels of plasma glucose, glycosylated hemoglobin and increased levels of insulin. Similarly, Kim et al. (2012) demonstrated that administration of tangeretin (200 mg/kg) can be increase insulin and glycogen secretion as well as decrease total cholesterol in high-fat diet (HFD)-induced obese mice. Neuroprotective activity of tangeretin was elucidated in an animal model of Parkinson's disease (PD) by Shu et al. (2014) that proved how tangeretin suppressed microglial activation in the LPS-stimulated primary rat microglia and BV-2 cell culture. Moreover, tangeretin inhibited metastasis in rat mammary carcinoma model through inhibition of matrix metallopeptidase 2 and 9 (MMP2 and MMP9) (Arivazhagan et al., 2014). Recently, Lee et al. (2018) proposed the application of tangeretin for the prevention or treatment of post-traumatic stress disorder (Patent no. KR102015221B1). This compound showed an excellent anti-anxiety effect, and consequently included in the pharmaceutical composition or food as an active ingredient. Tangeretin also, was as an active ingredient for alleviating, preventing or treating renal fibrosis or cirrhosis of kidney glomerulus or albuminuria (Young-hee & Min-kyung, 2018; Patent no. KR101949471B1). Ting et al., (2015) indicate that the limit of dosage responsible of mortality was higher than 3000 mg/kg bw after tangeretin oral administration in mice.

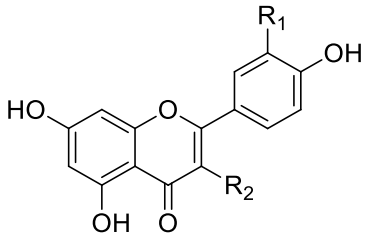
The polymethoxyflavone sinensetin is known as potent antioxidant, anticancer and anti-inflammatory agent with its beneficial actions against various metabolic diseases (Laavola et al., 2012). Mostly sinensetin act by inhibition of iNOS, NO production, COX-2 expression and prostaglandin E_2 (Laavola et al., 2012). Additionally, can inhibit LPS-induced inflammation by suppressing the expression of IL-1β, IL-6, and TNF-α genes associated with inflammation in macrophages (Yam et al., 2010). These results are in according with Kim et al. (2019b) that reported the anti-inflammatory activities of sinensetin on LPS-stimulated L6 skeletal muscle by regulating of NF-κB.

Kang et al. (2015) suggest that sinensetin may have potential as a natural agent for prevention/improvement of metabolic diseases such as obesity. In this study reported the effects of sinensetin on lipid metabolism in mature 3T3-L1 adipocytes without causing cytotoxicity in 3T3-L1 cells. This compound showed antiadipogenic property by downregulation of sterol regulatory element-binding protein 1c. Also, sinensetin increased the phosphorylation of protein kinase A and hormone-sensitive lipase, indicating its lipolytic property via a cAMP-mediated signalling pathway. Moreover, sinensetin inhibited insulin-stimulated glucose uptake by decreasing the phosphorylation of insulin receptor substrate and Akt. Furthermore, sinensetin increased the phosphorylation of AMPK and acetyl-CoA carboxylase. It also upregulated mRNA expression of carnitine palmitoyltransferase-1a, suggesting that sinensetin enhances fatty acid β-oxidation through the AMPK pathway. Additionally, the hypoglycaemic effects of sinensetin was investigated by Mohamed et al. (2012). Sinensetin showed strong inhibition percentages against α-glucosidase and showed IC_{50} values (0.66 mg/mL) significantly lower than acarbose (1.93 mg/mL). The same activity was observed against α-amylase with IC_{50} value of 4.89 mg/mL compared to acarbose (36.70 mg/mL).

Recently, Kim & Lee (2018) proposed the application of sinensetin as active ingredient for preventing, ameliorating, or treating liver cancer or gastric cancer (Patent no. KR20190050535A).

Luteolin, another *Citrus* polymethoxy flavanone, had its biological activities due its hydroxyl moieties and the double bond present between C2 and C3 (Sangheetha et al., 2019). In recent study, Sangeetha (2019) proposed that the antioxidant activity of luteolin coupled with its hypoglycaemic potential protects pancreas and promotes insulin secretion. The inhibition of free radical generation and repression of lipid peroxidation has been proved using experimental diabetic animal models. This report agreed with Xu et al. (2019) that proving how luteolin suppress oxidative damage, lipid peroxidation, and loss of antioxidant enzymes such as CAT and SOD. The antioxidant property of luteolin provides a basis for its use in treating neurodegenerative diseases. Some studies showed a great capacity of luteolin to protect neurons against oxidative stress. In fact, luteolin administration protected rats against the cognitive dysfunction and increased the neuron survival through reduction intracellular ROS accumulation together with restored level of MDA and the activity of GPx (Xu et al., 2014). The application of luteolin in inhibition of gastric secretion and reduction of pepsin activity was reported by Dai & Li (2018, Patent no. CN108309971B). In particular, the preparation includes 3-5 parts of luteolin and 1-2 parts of schisandrin B as active ingredients, and the dosage form of the compound preparation was preferably tablets, capsules, injections and granules. The oral LD_{50} in rats was > 5000 mg/kg bw (Kanai et al., 2016).

Compared with flavanones and flavones, the flavonol contents are much lower in *Citrus*. Flavonols are the 3-hydroxy derivatives of flavones (Figure 6). Glycosylation occurs preferentially at the 3-hydroxyl group of the central ring. The most common aglicones flavonols are quercetin and kaempferol, while rutin is the main one in the glycosidic form.



NAME	R_1	R_2
Quercetin	OH	OH
Isoquercetin	OH	O-β-D-glucopyranosyl
Kaempferol	OH	OH
Rutin	OCH_3	OH

Fig. 6. Flavonols structure

Quercetin has been used as nutritional supplement and may be beneficial effects against a variety of diseases. Several *in vitro* and *in vivo* studies evidenced its biological functions. More recently, Doustimotlagh et al. (2019) suggested the ability of quercetin, administered by injection, at a dose of 50 mg/kg/day for 10 days, in bile duct-ligated rats, to cause a significant decrease in protein carbonyl, hydroxyproline, and to regulate the GPx activity. Therefore, quercetin acts as an enzyme inducer by renewing the glutathione

peroxidase activity and inhibiting the oxidation of proteins and hence decrease ROS production. These results confirm the positive role of quercetin in attenuating the liver damage and degeneration. Milanezi et al. (2019) analysed the antioxidant activity of quercetin-capped gold nanoparticles. Quercetin-capped gold nanoparticles (IR_{50} 0.37 µg/mL) exhibited greater activity than free quercetin (IR_{50} 0.57 µg/mL) by NO free radical scavenging assay. Similarly, quercetin vesicular formulations (Eudragit-coated liposomes) were capable of ensuring optimal protection against oxidative stress in human intestinal cells by reducing ROS production, as reported by Caddeo et al. (2019). Its antioxidant capacities were correlated to the presence of two antioxidants pharmacophores in the molecule that had optimal configuration for free radical scavenging. The high antioxidant potential of quercetin was confirmed also in superoxide test with IC_{50} values of 0.025 mM *vs* 0.243 mM, for quercetin and kaempferol, respectively. Increasing *in vivo* studies have proved that quercetin acted as an antioxidant due to its ability to ameliorate antioxidant defences, decrease free radical formation, inhibiting xanthine oxidase, and inhibited lipid peroxidation (Shi et al., 2019). Previously, Lines (2003) reported the application of quercetin to treat Crohn's disease, arthritis, leukaemia, sexual dysfunction, chronic constipation, improving concentration or mood, lowering cholesterol levels and blood pressure (Patent no. EP1562447B1).

Literatures data showed that quercetin was able to reduce glucose levels when it was administered at the dose of 30 mg/kg bw for 14 days (Yang & Jang 2018), or at doses of 25-75 mg/kg for 28 days (Srinivasan et al., 2018), or at doses of 100 and 200 mg/kg bw for 6 weeks (Sharma et al., 2018). Additionally, this compound potentiated insulin secretion induced by glucose and glibenclamide and protected β-cells against oxidative damages (Shi et al., 2019). Eid et al. (2015) proposed the use of quercetin as anti-diabetic compound since this flavonoid can act through the stimulation of GLUT4 translocation in skeletal muscle and the inhibition glucose-6-phosphatase in hepatocytes. In a human study for 12-week, Lee et al. (2016) used 100 mg/day/subject of quercetin to treatment obesity and showed that this compound diminished the total body fat, and decreased the BMI of overweight or obese subjects. In addition, quercetin ameliorated mitochondrial functions in adipose tissue of HFD-induced obese mice by increasing the levels of oxidative stress-sensitive transcription factor and antioxidant enzymes (Kobori et al., 2016). A mixture of quercetin, myricetin and chlorogenic acid was registered as Patent no. CA2685031A1 for treating diabetes and metabolic disorders, and for achieving weight loss (Ahrens & Thompson, 2008). The acute oral toxicity of quercetin in mice was determined to be 575 mg/kg bw (Li et al., 2017).

Isoquercetin, called also quercetin-3-*O*-glucoside, is one of the naturally occurring glucosides of quercetin. This compound is recognized for its antiradical activity and thus to scavenge ROS and RNS, including superoxide anion radicals (Kim et al., 2013), hydroxyl radicals (Li et al., 2011), peroxyl radicals (Salucci et al., 2002) and peroxynitrite (Nugroho et al., 2014). Isoquercitrin is also found to scavenge superoxide radicals produced by a xanthine/xanthine oxidase system and to inhibit xanthine oxidase activity

itself (Salem et al., 2010). In rat peritoneal macrophages, this flavonoid was found to attenuate lipopolysaccharide (LPS)-induced expression of iNOS (Lee et al., 2008).

Additionally, this flavonol diminished H_2O_2-induced DNA damage in isolated human lymphocytes and decreased intracellular ROS levels, glutathione depletion and lipid peroxidation (Boligon et al., 2012). Investigation on possible anti-inflammatory activity shown that isoquercitrin may decrease the levels of prostaglandin E2 produced by LPS-stimulated RAW264.7 cells, presumably via inhibiting the activity of COX-2 (Valentová et al., 2014).

Isoquercitrin is also found capable to reduce the production of IL-6 in human osteosarcoma MG-63 cells stimulated by TNF-α (Kim et al., 2014a). Interesting effects of isoquercitrin on the cardiovascular system are also observed by Emura et al. (2007). After 22 days of oral administration this compound suppressed the increase in mean blood pressure and heart rate in spontaneously hypertensive rats. In the same model, isoquercitrin exhibited an antihypertensive effect involving the inhibition of the angiotensin-converting enzyme and a diuretic effect (Gasparotto Junior et al., 2012). Literature data on potential antidiabetic propriety revealed that isoquercitrin inhibited alloxan-induced hyperglycaemia, hepatic and renal lipid peroxidation and the activity of hepatic glucose-6-phosphatase, while the activities of catalase and SOD, and the content of glutathione were increased (Panda & Kar, 2007). This flavonol also delayed the glycaemic peak by 30 min in oral glucose tolerance tests and thus exhibited anti-hyperglycaemic activity. Additionally, α-glucosidase and α-amylase inhibition was observed (Valentová et al., 2014). The same authors reported that the oral LD_{50} in Sprague–Dawley rats was >25 g/kg. No clinical signs or mortality related to treatment were found.

Al-Numair et al. (2015) evaluated the protective effect of kaempferol against oxidative stress in STZ-induced diabetic rats. Kaempferol administration (100 mg/kg bw) to diabetic rats resulted in a reduction of plasma glucose, insulin, lipid peroxidation products, enzymatic such as SOD, CAT, GP_X, and GSTs. Additionally, in this study the oral LD_{50} value of kaempferol of 980 mg/kg in rats was reported. Previously, Vellosa et al. (2011) compared the antioxidant activity of quercetin and kaempferol through different assay. On scavenging hypochlorous acid quercetin was more efficient respect kaempferol with IC_{50} values of 0.0008 mM *vs* 0.0020 mM. A similar trend was reported in scavenging chloramines assay in which quercetin had an IC_{50} value lower than that reported for kaempferol (IC_{50} value of 0.044 mM *vs* 0.092 mM).

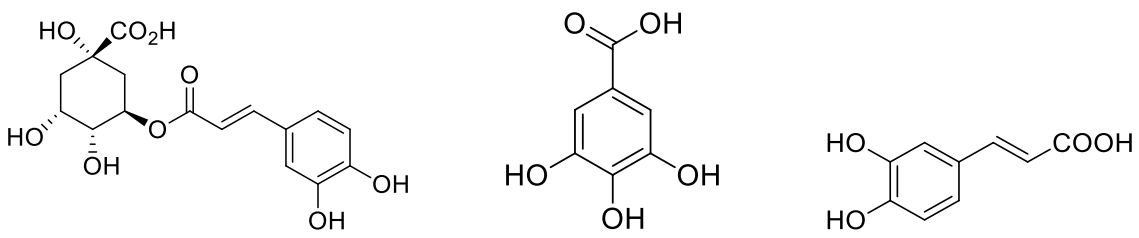
Another study, demonstrated that oral administration kaempferol (50 mg/kg/day than corresponding human equivalent dose of 240 mg/day for 60 kg), ameliorated blood glucose control in obese mice as well as reduced hepatic glucose production and improvement insulin sensitivity. Additionally, these authors found that kaempferol was a direct inhibitor of pyruvate carboxylase and suppressed gluconeogenesis in HepG2 cells (Alkhalidy et al., 2018). Torres-Villarreal et al. (2018) tested the effects of treatment with 60 μM of kaempferol for 21 days to evaluate its lipolytic and anti-adipogenic potential.

The results on anti-obesity effects showed that kaempferol modulated adipogenic differentiation in 3T3-L1 cells through promoting down-regulation of *Cebpa* gene expression and decreased lipid accumulation in mature adipocytes for its positive effects on *Pnpla2* and *Lipe* mRNA levels. In obese HDF-mice deficient in apolipoprotein E (ApoE), administration of kaempferol significantly reduced plasma glucose and triglyceride levels, increased high-density lipoprotein, cholesterol levels, and ameliorated insulin sensitivity and glucose tolerance (Chang et al., 2011). Similarly, rutin acted (Yuan et al., 2017) reducing adiposity, increasing energy expenditure, and improving glucose homeostasis in obese mice.

More recently, Kaurinovic et al. (2019) reported that rutin is a strong antioxidant agent for its ability to build chelates with metal ions and reduces Fenton's reaction in which harmful oxygen radicals was produced and potentiated the ascorbic acid activity. Ghorbani (2017) described anti-diabetic property of rutin. In STZ-induced diabetic rats, oral administration of 50 or 100 mg/kg of this compound decreased fasting blood glucose as well as HbA1c levels. Moreover, chronic administration of 200 mg/kg of rutin, determined a reduction (30-40%) of prevalence of diabetes in STZ-treated mice. In addition, more recently Gupta et al. (2019) demonstrated that rutin treatment (50 mg/kg) for 24 weeks arrested the biochemical disturbances of diabetic retinopathy lowering VEGF, TNF-α, and increasing total antioxidant capacity in the retina. The positive effect of rutin on lipid profile was reported by Wang et al. (2015). This compound decreased serum levels of triglycerides, very low-density lipoproteins, and increased the level of high-density lipoproteins in different models of DM. Additionally, rutin decreased ROS formation, advanced glycation end-product precursors, and inflammatory cytokines production. The anti-inflammatory activity of rutin was recently confirmed by Su et al. (2019). In this case, the inhibition of NF-κB pathway and understatement of endoplasmic reticulum stress was observed. Rutin have an oral LD_{50} of 160 mg/kg (Nakamura et al., 2000).

Phenolic acid

Phenolic acids have recently gained substantial attention due to their various practical, biological and pharmacological effects. In *C.* × *clementina* the most abundant phenolic acid was chlorogenic, caffeic and gallic acid (Figure 7) (Loizzo et al., 2018).



(a) (b) (c)

Fig. 7. Chemical structures of chlorogenic acid (a), gallic acid (b) and caffeic acid (c).

Chlorogenic acid is an important and bioactive dietary polyphenol, playing several important and therapeutic roles. Several studies reported the ability of chlorogenic acid to act in metabolic disease through different mechanisms of action. Recently, Kodimule (2018) proposed the use of chlorogenic acid in the treatment of metabolic disorders (Patent no. US20190111015A1). Diet supplementation with this compound at dose of 20 or 90 mg/kg bw for 12 weeks suppressed increases in serum lipid levels, while a dosage10 mg/kg significantly reduced total and LDL-cholesterol and increased HDL cholesterol by up-regulating the gene expression of PPAR-α in hypercholesterolemic rats (Huang et al., 2015; Wan et al., 2013). In *Lepr*$^{db/db}$ mice 250 mg/kg bw of chlorogenic acid intra-peritoneally administered for 2 weeks inhibited hepatic glucose-6-phosphatase expression and activity, decreased hepatic steatosis, improved lipid profiles and skeletal muscle glucose uptake, and ameliorated glucose levels, glucose tolerance and insulin sensitivity *via* the activation of AMPK (Ong et al., 2013). Additionally, administration of chlorogenic acid at the dose of 80 mg/kg BW for 12 weeks decreased percentage of body fat, fasting plasma glucose, and glycosylated haemoglobin (HbA1c) level *via* modulation of adiponectin receptor signalling pathways (Jin et al., 2015).

The anti-obesity effects of chlorogenic acid was observed in Male Sprague-Dawley rats at doses of 20 or 90 mg/kg bw for 12 weeks (Huang et al., 2015). Moreover, the treatment with chlorogenic acid (0.2 g/kg diet), or with caffeic acid (0.2 g/kg diet) for 8 weeks in obese mice, reduced body weight, plasma leptin and insulin levels, visceral fat mass, triglycerides and cholesterol levels (Cho et al., 2010). Oboh et al. (2015) evaluated the inhibitory effects of these phenolic acids on α-amylase and α-glucosidase activities. Data showed that caffeic acid had a higher inhibitory activity against α-amylase and α-glucosidase with IC_{50} values of 3.68 and 4.98 μg/mL, respectively compared to chlorogenic acid (IC_{50} values of 9.10 and 9.24 μg/mL, respectively for α-amylase and α-glucosidase). Additionally, the same authors suggested that both phenolic acids exhibited high antioxidant properties; caffeic acid showed higher effects with IC_{50} value of 38.83 μg/mL, while chlorogenic acid IC_{50} value of 9.10 μg/mL. Studies on the toxicity of chlorogenic showed that the oral LD_{50} for immature rats was greater than 1 g/kg (Meng et al., 2014).

More recently, Prudêncio et al. (2019) reported the antioxidant protection of caffeic and chlorogenic acid against oxidative stress *in vivo* using BY_{4741} strain and superoxide dismutase and glutathione-deficient mutants of *S. cerevisiae*. In the cell viability tests, caffeic acid showed higher stress tolerance, with a 106% increase in *S. cerevisiae* BY_{4741}. However, in the superoxide dismutase mutant, the effect of chlorogenic acid was stronger than caffeic acid, with a 3.3-fold increase. Conversely, in the glutathione-deficient mutant both treatments showed a similar level of protection. Recently, Arriagada et al. (2019) proposed the use of a hybrid nanocarrier consisting of core-shell silica nanospheres linked to the surface with caffeic acid. These nanospere are characterized by a potentiated antioxidant property respect the caffeic acid alone. It was reported a LD_{50} >721 mg/kg after intraperitoneal dose of caffeic acid in mouse (Yakugaku 1984).

Spagnol et al. (2019) reported that gallic acid presented an antioxidant activity greater than that reported for caffeic acid and ascorbic acid. Moreover, this acid had the advantage of a greater stability during extraction procedure by natural source. Gallic acid was able to restore vitamin C and GSH levels in the pancreas of STZ-treated rats. Moreover, this phenolic acid could exert its antioxidant activity trough different mechanism including free radical scavenging property, Fe^{2+} chelating and Fe^{3+} reducing abilities (Kade et al., 2014). Yang (2018) discloses a kind of anti-oxidant lightening compositions and its applications. The anti-oxidant lightening compositions include orange peel element (tangeretin) and gallic acid (Patent no. CN108464949A).

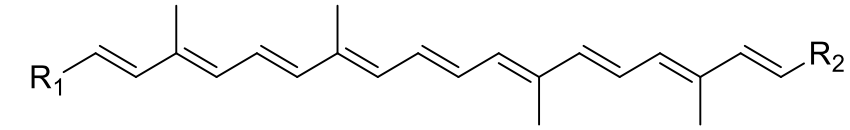
The protective role of gallic acid was also demonstrated on metabolic disorders. The antidiabetic effect of gallic acid administered at dose of 20 mg/kg determined the reduction in the size of adipocytes, protection of pancreatic β-cells by the induction of the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), and insulin sensitivity in adipocytes (Gandhi et al., 2014). Additionally, the same study reported the capacity of this compound to increase the cellular glucose uptake by activation of GLUT4 translocation. The intraperitoneal treatment with gallic acid, at dose 10 mg/kg/day for 2 weeks, ameliorated the triglyceride and the blood glucose level in the obesity mice by an up-regulation of PPARγ expression and Akt activation (Bak et al., 2013). Previously, Punithavathi et al. (2011) reported that gallic acid (10 and 20 mg/kg for 21 days) scavenge free radicals and improved the activities of SOD, CAT. The inhibition of lipid peroxidation was also observed. More recently, Przybylska-Balcerek et al. (2019) reported that oral toxicity of gallic acid (5000 mg/kg bw in rat).

Carotenoids

Carotenoids are lipid-soluble pigments, coloured from yellow to red, with a basic structure consisting in a tetraterpene with a series of conjugated double bonds. The most abundant *C.* × *clementina* carotenoids are β-cryptoxanthin, lutein, violaxanthin and β-carotene (Figure 8) (Poiroux-Gonord et al., 2012).

In humans, carotenoids were recognized for their biological activities associated with the reduction of the risk of developing chronic diseases such as cancer, cardiovascular and neurodegenerative diseases as well as metabolic disease. Additionally, these compounds acted as antioxidants and protected the cells against free radicals formed in the tissues. Some of these compounds were vitamin A precursor (Cardoso et al., 2016).

β-Carotene is an intensely-coloured orange pigment, used as a food colouring agent with the E number E160 (Milne 2005). In nature, β-carotene is a precursor (inactive form) of vitamin A, which is synthesized from carotenoids *via* the action of the enzyme β-carotene 15,150 -monooxygenase. Asemi et al. (2016) investigated the beneficial effects of β-carotene fortified synbiotic food intake on metabolic status in T2DM patients. The β-carotene fortified synbiotic food contain also *Lactobacillus sporogenes* (1×10^7 CFU), 0.1 g inulin and 0.05 g β-carotene. Results showed that this synbiotic food had favorable effects on homeostatic model assessment of insulin resistance, insulin, triglycerides, VLDL-cholesterol, total-/HDL-cholesterol *ratio*, NO and glutathione levels.



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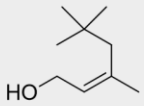
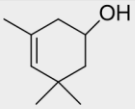
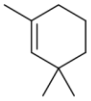
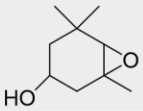
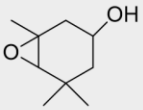
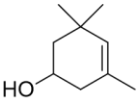
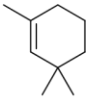
		Lutein
		β-carotene
		violaxanthin
		β-cryptoxanthin

Fig. 8. Carotenoids structure

Torregrosa-Crespo et al. (2018) reported the antioxidant immune response, anti-inflammatory, anti-diabetic and antitumor activity of β-carotene. The antioxidant activity was correlated to its capacity to inhibits Na^+K^+-ATPase enzyme and stimulate CAT and GSTs (Raposo et al., 2014). Ben Amara et al. (2015) suggested the existence of a positive effect of β-carotene on insulin sensitivity in obese patients through a positive regulation of adiponectin, either directly or *via* its pro-vitamin A activity. Moreover, β-carotene reduces body adiposity (28%) of mice *via* $Bcmo^{1-/-}$ (Amengual et al., 2011).

β-carotene is not toxic but the high and constant administration of this substance can translate into skin yellow coloration, and the registered LD_{50} is >5000 mg/kg (Dasgupta & Klein, 2014). Recently, Chaudhury & Talapatra (2019) reported value of LD_{50} of 8032.73 mg/Kg in rat after oral administration.

Lutein, (β,ε-carotene-3,30-diol), acted as a powerful antioxidant, prevented high fat diet-induced atherosclerosis in apoE-deficient mice by inhibiting NADPH oxidase and increasing PPAR-α expression (Han et al., 2015). Additionally, it protects dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced apoptotic death and motor dysfunction by ameliorating mitochondrial disruption and oxidative stress (Nataraj et al., 2016). Administration of lutein at dose of 1.5 mg/kg, in STZ-induced diabetic rats showed kidney protective effects correlated to its antioxidant property by preventing ROS overproduction and increasing the removal of preformed ROS (Katyal et al., 2013). Acute toxicity study revealed that the LD_{50} exceeded the highest dose of 10000 mg/kg bw in mice after oral administration (Nidhi & Vallikannan Baskara, 2013). β-Cryptoxanthin is used as colouring agent for food products in certain countries and is associated with the E number E161c. β-Cryptoxanthin, obtained from its

common food sources, exhibited high bioavailability and β-cryptoxanthin-rich foods might be considered equivalent to β-carotene-rich foods as sources of retinol (Burri et al., 2016). Ghodratizadeh et al. (2014) demonstrated that β-cryptoxanthin (5-10 mg/kg) increased serum IgG, IgM IgA and IL-4 levels in rabbit. The cancer preventive effects of β-cryptoxanthin were described by Leoncini et al. (2016). The study included over 6000 subjects with oral, laryngeal, and pharyngeal cancer. The treatment with β-cryptoxanthin determined a reduction of at least 18% in the rate of oral and pharyngeal cancer and a 17% reduction in the rate of laryngeal cancer. Acute toxicity study revealed that the LD_{50} is 3798mg/kg bw in mice after oral administration (Chaudhury & Talapatra, 2019).

Violaxanthin is a natural xanthophyll pigment, orange in colour and was used food additive, associated with E161e. Soontornchaiboon et al. (2012) inhibited NO production, the prostaglandin E_2 (PGE_2), by interfering with LPS-mediated NF-κB pathways.

Coumarins and furanocoumarins

Coumarins (benzo-α-pyrones) represent a class of secondary metabolites commonly found in a limited number of plant families, such as Rutaceae, Apiaceae, Moraceae, and Fabaceae. Umbelliferone (7-hydroxycoumarin), is a ubiquitous coumarin of higher plants that can undergo subsequent biochemical modifications corresponding to a prenylation step at C6 or C8 with generation of furanocoumarins.

These compounds are characterized by the presence of an additional furan ring linked at the C6/C7 (linear type) or C7/C8 (angular type) position of the coumarin nucleus. The most abundant coumarins found in *C.* × *clementina* are limettin and auraptene (Figure 9).

The furanocoumarins isopimpinellin, bergapten, bergamottin and oxypeudanin were also identified (Dugrand-Judek et al., 2015).

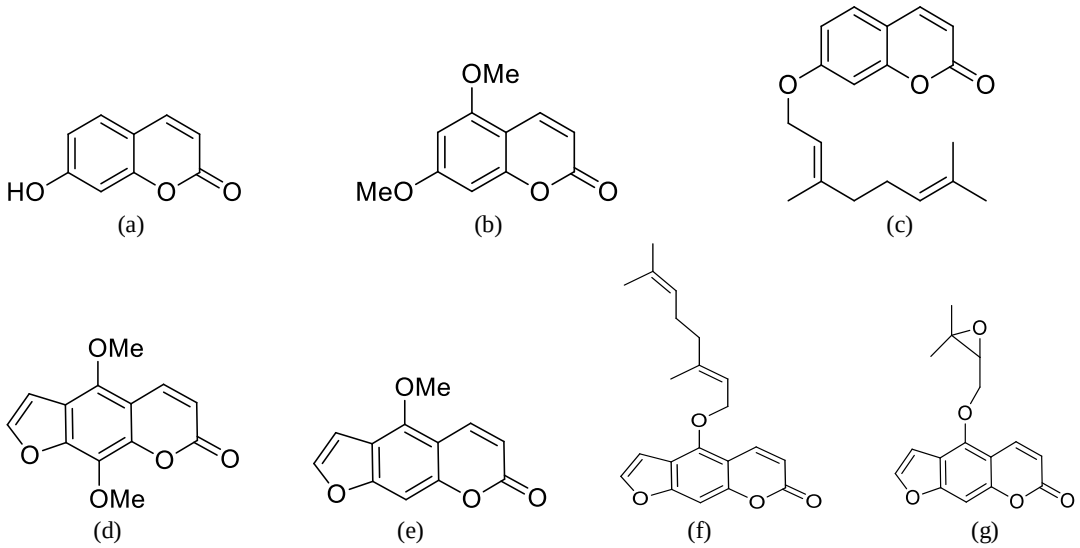


Fig. 9. Umbelliferone (a), limettin (b), aurapten (c), isopimpinellin (d), bergapten (e), bergamottin (e), and oxypeucedanin (g)

Furanocoumarins showed several biological activities such as antioxidant, anti-inflammatory, anti-proliferative, and as promoters of bone health. These effects making

them the focus of a great number of investigations for both academic and industrial researchers (Bruni et al., 2019). The linear furanocoumarins were historically used for treatment of skin disorders, such as psoriasis, vitiligo, polymorphous dermatitis, eczema, and mycosis (Sarker et al., 2004).

Kim et al. (2014b) reported the antiproliferative effects on breast cancer cell growth of bergamottin, bergaptol, and bergapten. Treatment with bergamottin at dose of 100 μM for 6 h of MDA-MB-231 breast cancer cells caused a significant reduction of phosphorylation, nuclear translocation, expression, and DNA binding activity of signal transducers and activator of transcription 3 (STAT3) (Bruni et al., 2019). In addition, administration of bergamottin and simvastatin in patients with chronic myelogenous leukaemia potentiated the inactivation of NF-kB expression and the induction of apoptosis (Kim et al., 2016b). More recently, Ham et al. (2019) demonstrated that bergapten (0.02%, w/w) prevented diabetic osteoporosis by suppressing bone resorption. In fact, it was observed a significant increase of bone volume density, trabecular number and a down-regulation of osteoclast-related genes with reductions of nuclear of activated T-cells, cytoplasmic 1 and tartrate-resistant acid 5 expression. Panno et al. (2012) and De Amicis et al. (2015) suggested that bergapten in a dose-dependent manner inhibited both MCF-7 and ZR-75 cells growth by apoptotic pathway (activation of p53 and caspases).

Moreover, in tamoxifen-resistant MCF-7, this furanocoumarin reduced the amount of estrogen receptors through the blocking of the protein transduction process and the depletion of the same receptor through SMAD4-mediated ubiquitination. Additionally, in MCF-7 and ZR-75 breast cancer cells, treatment with bergapten activated the expression of Beclin, UV radiation resistance-associated gene (UVRAG), and activating molecule in Beclin-1-regulated autophagy (AMBRA), and subsequently significantly increased autophagy. The same study has also found that phosphatase and tensin homologue deletion on chromosome 10 as being mainly responsible for bergapten activity in the induction of breast cancer cells autophagy. Oxypeucedanin showed antiproliferative activity against HeLa cells with an IC_{50} value of 314.0 μg/mL. This compound could be act also as antioxidant, antiarrhythmic, antibacterial, antifungal, and antiestrogenic activity (Razavi et al., 2010). Shalaby et al. (2014) reported the anti-diabetic activity of furanocoumarins that acted *in vitro* as α-amylase, α-glucosidase, and α-galactosidase inhibitors. Bergapten and imperatorin possessed the highest inhibitory activity in comparison to oxypeucedanin hydrate and psoralen.

In parallel to their beneficial proprieties, these compounds have controversial effects on humans, acting both as potential photosensitizers and due their ability to interact with several drugs. When irradiated with UV light, furocoumarins can undergo photoactivation, form adducts with DNA, induce protein denaturation, form cyclo adducts with saturated fatty acids and form ROS (Dugrand-Judek et al., 2015). In particular, a contact with *Citrus* plants molecules may cause dermatitis, hyperpigmentation and blisters due the presence of bergapten, psoralen and xantotoxin. Previously, Brickl et al. (1984) determined that the lowest dose of xantotoxin combined with UVA was 14 mg (corresponding to about 0.23 mg/kg bw for a 60 kg adult).

Subsequently, Schlatter et al. (1991) established that a combined dose of 10 mg xantotoxin and 10 mg of bergaptene (0.25 mg/kg bw for a 60 kg adult) was equivalent to 15 mg of xantotoxin dose. While, only a few studies addressed the phototoxic effects due to the consumption of beverages or foods containing furanocoumarins. Gorgus et al. (2010) reported that in the Western diet, exposure to furanocoumarins could mainly derive from grapefruit juice and also determined that the amount of grapefruit juice containing 20 mg of bergamottin was 1.2 L. Consumption of such amount of grapefruit juice is unrealistic and therefore the phototoxic effects are unlikely. Further controversial effects of furanocoumarins are related to the ability to inhibit the intestinal cytochrome P450-3A4 also called CYP3A4 through inhibition of basic mechanisms. For patients undergoing drug therapy, the inhibition of cytochrome P450 by furanocoumarins may lead to a higher concentration of drug in the blood, which in turn can cause serious side effects such as heart rhythm disturbances or respiratory depression (Dugrand-Judek et al., 2015). This effect is often referred to as "the effect of grapefruit juice". Masuda et al. (2018) reported the capacity of grapefruit juice to increase the oral availability of nifedipine, cyclosporin A, midazolam, triazolam, and tacrolimus. The CYP3A4 inhibition was mainly attributed to bergaptol and its derivatives such as bergaptene, isoimperatorin, and bergamottin.

Terpenes

Terpenes are naturally occurring substances found in essential oils of plants and are responsible for a plant's distinct fragrance. Essential oils are mixture of different organic substances, volatile, characterized by a strong odour, rarely coloured, obtained by cold pressing, hydrodistillation, steam current distillation, supercritical fluid extraction, enfluorage, and extraction with volatile solvents using plant organs such as f flowers, leaves, buds, seeds, bark, herbs, and root stored in secretory cells or epidermal cells.

The aroma, that characterizes each oil, is the combination of the aromas of all the components including those present in smaller quantities. This phytocomplex represents a small fraction of plant’s composition and can be used in the food, cosmetic and pharmaceutical industries (Miguel, 2010).

Monoterpenes are secondary metabolite with various pharmacological properties including antifungal, antibacterial, antioxidant, anticancer, anti-spasmodic, hypotensive, and vasorelaxant. In addition, showed a protective effect on the cardiovascular system, acting as a vasodilator and reducing heart rate and hypotension (Santos et al., 2011). Sabinene, limonene, and δ-3-carene (Figure 10) represented the major constituents of *C.* × *clementina* EO (Nguyen et al., 2015).

Sabinene was found to be a potential modulator of bacterial resistance. This monoterpene could be act in synergism with antibiotics to reduce MIC values against bacterial strains PA03 and SA358 (Matias et al., 2016). Previously, Valente et al. (2013) reported the antimicrobial activity of sabinene against *Trichophyton* strains with MIC values ranging from 0.16 to 0.32 μL/mL followed by *Microsporum gypseum* (MIC= 0.32-0.64 μL/mL), *Candida* (MIC= 0.64-1.25 μL/mL) and *Aspergillus* strains (MIC= 1.25-

2.5 μL/mL). Additionally, the same research group demonstrated sabinene anti-inflammatory activity *via* NO production inhibition (135.6%). The anti-inflammatory potential can be related to its scavenging ability and/or capacity to inhibit iNOS expression. In fact, sabinene exhibited scavenging activity at concentrations of 0.64 μL/mL. This result agreed with Dorman (2000) that reported the relative antioxidant capacity of 14.76 TE (mmol/L) for the same compound.

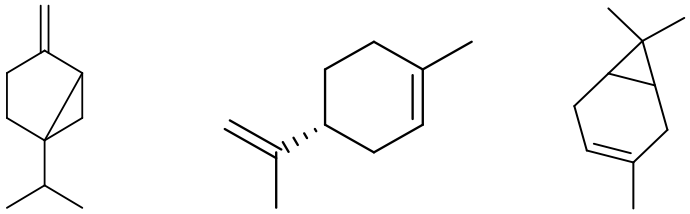


Fig. 10. Sabinene (a), limonene (b) and δ-3-carene (c),

D-Limonene is a monocyclic terpene consumed by humans as an ingredient of traditional foods and it is listed in the Code of Federal Regulations as generally recognized as a safe (GRAS) flavouring agent (Roberto et al., 2009).

Recently, Verma et al. (2019) conduced *in vitro* studies on ameliorative effects of limonene on cadmium-induced genotoxicity in cultured human peripheral blood lymphocytes. The data showed that this terpene at concentration of 20 and 100μM reduced the sister chromatid exchange frequency and peroxidation of lipids. Previously, Murali et al. (2012) evaluated the protective effects of d-limonene on the levels of lipid peroxidation and antioxidant defences systems in the plasma and tissues of STZ-induced diabetes rats.

Administration of D-limonene (100 mg/kg bw) to diabetic rats for 45 days caused a significant reduction in the levels of lipid peroxidation by-products and an increase in the activities of antioxidant enzymes such as DOD, CAT, GPx and GSTs, when compared with the untreated diabetic group. There was no significant difference in normal treated groups, when compared with normal rats.

Limonene, is made up of two isoprene units, and the presence of two double bonds renders it a potential antioxidant compound. These results agreed with those reported by Yu et al. (2017) that observed how treatment with 50 or 100 mg/kg of d-limonene, increased endogenous antioxidant enzymes level. Souza et al. (2018), suggested that treatment with limonene (50 mg/kg) displayed anti-inflammatory activity through decreasing TNF-α, IL-6, and IL-1β levels and increasing the level of IL-10. Additionally, this compound determined 93% of reduction in gastric ulcer area and higher preservation of gastric mucosa integrity by reducing myeloperoxidase (MPO) activity as well as an increase in GPx activity that suggested an antioxidant effect of this compound. Tang et al. (2019) reported that d-limonene protected PC12 cells against corticosterone-induced neurotoxicity by activating the AMPK pathway. In fact, it was observed a reduction of

MDA and NO levels, NADPH oxidase activity, iNOS, COX-2, IL-6, IL-1β, TNF-α, and expression of pro-apoptotic proteins.

δ-3-Carene is bicyclic monoterpene hydrocarbon and it is recognized for its biological proprieties. In particular, δ-3-carene showed IC_{50} value of 0.603 mg/mL in thiobarbituric acid reactive substances (TBARS) test (Aazza et al., 2011) and 3.93 mmol TE/L in ORAC test (Dorman et al., 2000). The neuroprotective activity of δ-3-Carene was investigated by Aaza et al. (2011). Authors evidenced that this bicyclic monoterpene hydrocarbon acted as strong AChE inhibitor with IC_{50} value of 0.0358 mg/mL.

Both linalool and terpinen-4-ol (Figure 11), members of oxygenated monoterpenes class, were found in *C.* × *clementina* (Nguyen et al., 2015).

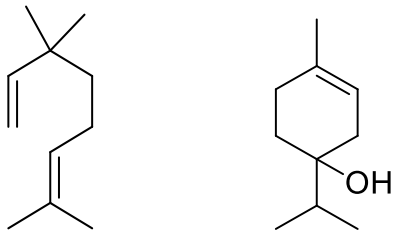


Fig. 11. Linalool (a) and terpinen-4-olo (b)

Linalool is an acyclic monoterpene tertiary alcohol and it is one of the most investigated aroma compounds. Several studies reported its biological activities. This compound acted as an anti-lipoperoxidant agent at concentrations of 120mg/kg and showed a protection of guinea-pig brain tissue against hydrogen peroxide oxidative stress, with similar effects to vitamin E (Aprotosoaie et al., 2014). Previously, Dorman et al. (2000) reported a relative antioxidant capacity of 14.29 mmol TE/L. Moreover, linalool had sedative effect due its ability to modulate glutamatergic neurotransmission.

Linalool behaved also as a spasmolytic agent on the intestinal and tracheal smooth muscles through adenylate cyclase enzyme stimulation and consequently an increase of cAMP (Aprotosoaie et al., 2014). This compound inhibited significantly and in a dose-dependent manner the LPS-induced production of TNF-α and IL-6 cytokines (Huo et al., 2013). In addition, linalool caused in mice a reduction of total cholesterol, LDL-cholesterol and triglycerides plasmatic levels. The same activity was noted in human hepatoma HepG2 cells. Molecular mechanisms involved the suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) transcription (reducing the expression of sterol regulatory element binding proteins) and the acceleration of HMGCR ubiquitin-dependent degradation.

At concentrations of 0.1%, linalool exhibited antimicrobial activity against different strains such as *S. aureus*, *E. coli*, *Bacillus subtilis* and *Pasteurella multocida* with major activity against Gram-positive bacteria than that to Gram-negative bacteria. The antibacterial activity was correlated to functional destabilization of bacterial membrane

with an increase susceptibility to the action of classical antimicrobial agents (Aprotosoaie et al., 2014).

The antibacterial activity was demonstrated also for terpinen-4-ol. Recently, Dore et al. (2018) described the efficacy of this compound in preventing both clinical and subclinical mastitis in sheep. Previously, Mondello et al. (2006) suggested that terpinen-4-ol showed MIC_{90} value of 0.06% against azole-susceptible and -resistant human pathogenic *Candida* species. The antioxidant capacity of 7.62 mmol TE/L was demonstrated by Dorman (2000).

Nguyen et al. (2015) reported β-caryophyllene and β-farnesene (Figure 12) as main sesquiterpens hydorcarbons of *C.* × *clementina*.

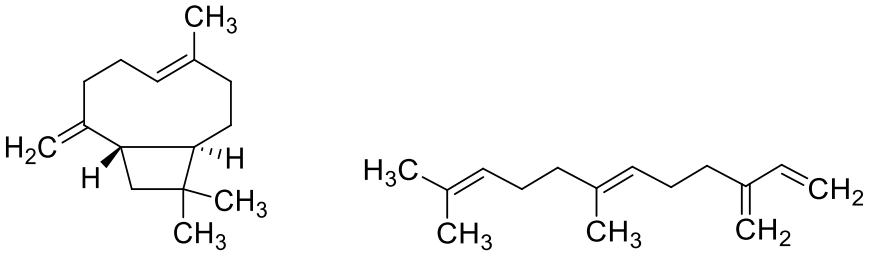
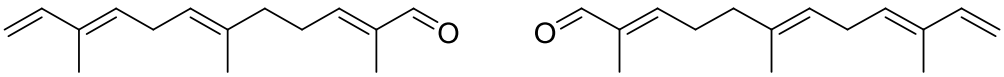


Fig. 12. β–Caryophyllene (a) and β-farnesene (b)

Baldissera et al. (2017) evaluated the effect of β-caryophyllene on hypercholesterolemia in rats and the possible effect on hepatic antioxidant enzymes. β-Caryophyllene administration at dose of 1.0 mL/kg for 3 days reduced the levels of total cholesterol, LDL cholesterol and triglycerides, inhibited the HMG-CoA reductase activity and increased the antioxidant system on ROS and TBARS levels.

These results agree with Basha & Sankaranarayanan (2016) that investigated the effect of β-caryophyllene on hyperglycaemia. Oral administration of this compound (200 mg/kg bw) for 45 days determined a reduction of glucose and increase of insulin levels, with restore antioxidant status enhancing the activity of CAT, SOD, GPx as well as inhibition of pro-inflammatory cytokines, TNF-α and IL-6. More recently, Hu et al. (2017) demonstrated that β-caryophyllene reduced prostaglandin E_2, iNOS production and COX-2 expression. Successively, Varga et al. (2018) evidenced that at 10 mg/kg dose this compound improved the chronic and binge alcohol-induced liver injury and inflammation through attenuating the pro-inflammatory phenotypic `M1` switch of Kupffer cells and diminishing the expression of E-Selectin, P-Selectin and neutrophil infiltration. Additionally, it ameliorated the hepatic metabolic dysregulation such as, protein hyperacetylation, steatosis, and PPAR-α signaling. These protective effects were correlated to activation of type 2 cannabinoid receptor (CB2). The interaction with CB2 receptor causes also the expression of vascular cell adhesion molecule-1 (VCAM-1) was mediated by the JAK2/STAT1/IRF-1 pathway (Zhang et al., 2017).

α-Sinensal and β-sinensal (Figure 13) represented the oxygenated sesquiterpens found in this fruit (Nguyen et al., 2015)



(a) **(b)**

Fig. 13 α–Sinensal (a) and β-sinensal (b)

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Chapter 3
Materials and methods

3.1 Chemicals and reagents

Solvent of analytical grade was obtained from VWR International s.r.l. (Milan, Italy). Tween 20, ascorbic acid, Folin-Ciocalteu reagent, sodium carbonate, butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), tripyridyltriazine (TPTZ), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS) solution, β-carotene, linoleic acid, Orlistat, Trizma base, 4-nitrophenyl octanoate (NPC), maltose, α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae*, *O*-dianisidine dihydrochloride, and PGO enzyme preparation were purchased from Sigma-Aldrich S.p.a. (Milan, Italy). Acarbose from *Actinoplanes* sp. was obtained from Serva (Heidelberg, Germany). Caffeic acid (PubChem CID: 689043), Protocactechuic acid (PubChem CID: 72), *p*-coumaric acid (PubChem CID: 637542), Chlorogenic acid (PubChem CID: 1794427), Vanillic acid (PubChem CID: 8468), Eriocitrin, (PubChem CID: 83489), Gallic acid (PubChem CID: 370), Apigenin (PubChem CID: 5280443), Dydymin (PubChem CID: 16760075), Quercetin (PubChem CID: 5280343), Hesperidin, (PubChem CID: 10621), Neohesperidin (PubChem CID: 232990), Neoeriocitrin (PubChem CID: 114627), Naringin (PubChem CID: 932), Narirutin (PubChem CID: 442431), Sinensetin (PubChem CID: 145659), Tangeretin (PubChem CID: 68077), Rutin (PubChem CID: 5280805), Isoquercetin (PubChem CID: 25203368), Genistin (PubChem CID: 5281377), Poncirin (PubChem CID: 442456), Luteolin (PubChem CID: 5280445), Kaempferol (PubChem CID: 5280863), Hesperetin (PubChem CID: 72281), Rhamnetin (PubChem CID: 5281691), Umelliferone (PubChem CID: 5281426), Isopimpinellin (PubChem CID: 68079), Bergapten (PubChem CID: 2355), were purchased from Sigma-Aldrich Chem. Co. (Milwaukee, WI, USA). Acetonitrile, formic acid, methanol and water were solvent HPLC grade, obtained from Carlo Erba Reagents (Milano, Italia).

3.2 Plant materials and extraction procedure

The fruits of *C.* × *clementina* Hort. (“Clementine di Calabria”, PGI) (Rutaceae) used in this study were collected in November 2016 in Calabria (Southern Italy), Plain of Sybaris (39°45′00″ N / 16°25′22.8″ E,100 m above the sea) in three different areas (A, B and C), Cetraro (39°30′59″ N / 15°56′29″ E, 138 m above the sea), Rosarno (Latitude: 38°29′13″ N, Longitude: 15°58′46″ E, 68 m above the sea) and Corigliano Calabro (Latitude: 39°35'45"60 N, Longitude: 16°31'6"60 E, 210 m above the sea).

These fruits were washed to remove superficial contamination, dried with paper, peeled, squeezed, and the juices (JA, JB, JC JD, JE, and JF from Sibari, Cetraro, Rosarno, and

Corigliano Calabro, respectively) were collected in a separate container and stored at 4 ◦C for further analysis.

C. × *clementina* leaves and peels were collected in November 2017 and 2018 respectively, in Calabria (Southern Italy). These samples were blended and extracted with different methodologies, such as maceration, Soxhlet apparatus, ultrasound-assisted extraction, and hydrodistillation. In particular, polar extracts were obtained by: *a)* Soxhlet apparatus using ethanol (1:14 g/mL, 7 cycles), *b)* maceration using EtOH (1:6 g/mL, 3 × 72 h) and 80% v/v hydroalcoholic solution of ethanol (1:6 g/mL, 3 × 72 h), *c)* ultrasound-assisted maceration using EtOH (1:7 g/mL, 3 × 1 h), and 80% v/v hydroalcoholic solution of ethanol (1:7 g/mL, 3 × 1 h). For this extraction procedure three extraction cycles with an ultrasonic frequency of 40 kHz at a temperature of 30 °C for 60 min were conducted for each sample in a Branson model 3800-CPXH water bath (Branson, Milan, Italy). After each extraction cycle, the mixture was filtered through Whatman filter Paper 4 under vacuum, and the solvent was removed using a rotary vacuum evaporator at 30 °C. Each extraction was performed in triplicate.

The authentication was carried out at the Natural History Museum of Calabria and the Botanic Garden, University of Calabria by Dr. Nicodemo Passalacqua.

Essential oils (Eos) were obtained by hydrodistillation of fresh leaves and peels for 3 h using a Clevenger-type apparatus (Clevenger, 1928). A white-yellow essential oil was obtained. The oil was dried over anhydrous sodium sulphate, stored in hermetically sealed brown glass bottles, and kept at 4°C before analysis.

Selected dried extracts were added to *C.* × *clementina* juice to obtain final concentrations of 20, 15,10, 5% w/v. Enriched juices were stored at 4 °C.

In order to evaluate the impact of food processing on the phytochemical content and bioactivity of the functional juices, 20% w/v enriched juice were pasteurized as reported by Rabie et al. (2014). The fresh juices (250 mL) were poured into dark jars inside and heated at 90 ◦C for 10 min using a thermostatic water bath (Branson model 3800-CPXH, Milan, Italy). Pasteurized juice was cooled to room temperature in a water bath for 30 min.

3.3 *C.* × *clementina* fruits quality parameters

Twenty-five fruits for each area of growth (Sibari, Cetraro, Rosarno and Corigliano Calabro) were collected and examined for integrity and absence of insect and dust contamination. Physical characteristics of the fruits such as fruit weight (g), equatorial diameter (cm), longitudinal diameter (cm), fruit firmness (g/0.5 cm^2), peels thickness (mm), total seeds per fruit and amount of extracted juice (%) were determined. Samples were freeze-dried and stored at −20 °C until analysis. Ash content, fat content, crude fiber content, total carbohydrates, and energy values were evaluated (Loizzo et al., 2018).

3.4 *C.* × *clementina* juice quality parameters and sensory analysis

C. × *clementina* fruits were squeezed, and the juice was centrifuged and filtered by Whatman #54 filter paper for analysis.

Color of fresh juice was measured at 25 °C using a Konica Minolta CM-700/600d spectrophotometer (Konica Minolta Sensing, Japan). Data were expressed as higher saturation of color or chroma (C*). C* considered as the quantitative indicator of colorfulness, is used to determine the degree of difference in a hue in comparison to a grey color with the same lightness. The higher the chroma values, the higher color intensity of samples is perceived by humans. Hue angle (h*), considered the qualitative indicator of color, is an attribute according to which colors have been traditionally defined as reddish, greenish, and is used to define the difference of a certain color with the reference to grey color of the same lightness. This attribute is related to the differences in absorbance at different wavelengths. A higher hue angle represents a lesser yellow character in the assays. An angle of 0° or 360° represents red hue, whilst angles of 90°, 180° and 270° represent yellow, green and blue hues, respectively (Pathare et al., 2013).

Total soluble solids (TSS) were determined using a digital refractometer PR-201α (Atago, Tokyo, Japan), previously calibrated at 20 °C and the results are expressed as degrees Brix (°Brix).

The pH was measured at ambient temperature with a pH meter (Model Basic 20, Crison) previously calibrated with standard solutions pH 4 and pH 7.

Total acidity (TA) was determined using the International Federation of Fruit Juice producers test (IFU): a potentiometric titration of the acidity of the juice, with a solution of 0.25 N NaOH up to pH 8.1. The results were expressed as g citric acid/100 mL. Ascorbic acid was determined using the International Federation of Fruit Juice producers test (IFU): a potentiometric titration of the acidity of the juice, with a solution of 2,6-dichloroindophenol (Loizzo et al., 2018). All determinations above described were made in triplicate.

The mineral elements in *C.* × *clementina* pulp were measured using AAnalyst 600 atomic absorption spectrophotometer with flame atomisation (Perkin Elmer, Milan, Italy). The measurements were made in hold mode with air acetylene flame. To achieve maximum sensitivity and precision, the equipment was equilibrated by alignment of the lamp and lighter and adjustment of the selected wavelength. The analytical conditions for the measurement of mineral elements were established using the respective acidified standard. The charred pulp was then ashed in a muffle furnace at 550◦C until a whitish ash was obtained. The ash was treated with 5 mL of nitric acid 5 N, transferred to a volumetric flask and made up to 100 mL. All standard solutions were measured using sodium, potassium, magnesium, and a calcium hollow cathode lamp at respective wavelengths of 589, 766, 285, and 422 nm using air acetylene flame.

Sensory evaluation was conducted by a selected and trained panel comprising 15 judges from graduate students of Science of Nutrition. Samples were served at 12-15 °C in tasting glasses and coded. Each subject received 17 samples (unidentified, with randomly assigned three-digit codes): a control juice sample (without dried extracts) pasteurized (JPF) and no pasteurized (JF), enriched juice with different extract concentrations (20, 15, 10, and 5% w/v) and pasteurized enriched juice (20% w/v). The evaluation was done using 9-point structured scales, 9 being the best and 1 the worst

quality (colour, odour, appearance, aroma, sweetness, acidity, astringency, and mouthfeel).

3.5 Gas chromatography mass spectrometry (GC-MS) analyses

Essential oils were subjected to analysis by gas-chromatography associated with mass spectrometry (GC-MS), using a Hewlett-Packard gas chromatograph equipped with a non-polar HP-5 capillary column (30 m × 0.25 mm, 0.25 μm), associated with a Hewlett-Packard mass spectrometer. The ionization of the sample constituents was performed in electronic impact (EI, 70 eV). The analyses were carried out with the following temperature schedule: isotherm at 50 °C for 5 minutes, temperature increase from 50 to 250 °C of 5 °C/min, and finally isotherm at 250 °C for 10 minutes. Helium is used as carrier gas. The identification of compounds was based on the comparison of the mass spectral data with the Wiley 138 library and referring to the spectral data of pure standards and compounds known in the literature. Essential oils were also analysed using a Shimadzu GC17A gas chromatograph (GC), equipped with an HP-5 capillary column (30 m × 0.25 mm, 0.25 μm). Nitrogen was used as transport gas. The conditions used are the same as those described for the GC-MS analyses.

3.6 Total phenols, flavonoids and carotenoids content

C. × *clementina* total phenols content (TPC) was evaluated by using the Folin-Ciocalteu method as previously reported by Gao et al. (2000). The sample at concentration of 1.5 mg/mL (0.1 mL) was mixed with a solution of Folin-Ciocalteu reagent (0.5 mL) and water (1 mL). After one minute of incubation 1.5 mL of 20% sodium carbonate was added and mixture was incubated at room temperature. The absorbance was measured at 765 nm using a UV-Vis Jenway 6003 spectrophotometer (Carlo Erba, Milan, Italy). The total phenols content was expressed as mg of chlorogenic acid equivalents (CAE)/g of fresh weight (FW).

Total flavonoids content (TFC) was determined spectrophotometrically using a method based on the formation of a flavonoid-aluminium complex (Loizzo et al., 2018). The extract (1.5 mg/mL) was mixed with aluminium chloride solution (2%) in a ratio 1:1 and incubated at room temperature for 15 min. The absorbance was measured at 510 nm. The total flavonoids content was expressed as mg quercetin equivalents (QE)/g FW.

The total carotenoids content (TCC) was determined as previously described (Loizzo et al., 2018). Briefly, 1 mL of the extract was added to 0.5 mL of NaCl 5% solution, vortexed for 30 seconds and centrifuged at 4500 rpm for 10 min. The supernatant (100 μL) was diluted with 0.9 mL of n-hexane and measured at 460 nm. The total content of carotenoids was expressed as equivalent mg of β-carotene/g FW.

3.7 HPLC analysis

3.7.1 Samples phenolic profile

High performance liquid chromatography (HPLC) was employed in order to investigate the *C.* × *clementina* phenolic profile. The HPLC analysis was performed using

a Knauer instrument (Asi Advanced Scientific Instruments, Berlin, Germany) and a UV-Vis diode array detector (DAD). The individual compounds were quantified by direct injection of the samples, appropriately diluted in the mobile phase and filtered through a 0.45 µm filter (Sartorius Minisart RC-4) in an HPLC system (Knauer Smartline Pump 1000), equipped with a Knauer Smartline UV detector 2600, and using a Knauer column Eurospher 100-5 C18 (150 × 4.6 mm equipped with a guard column) at 25 ◦C.

The solvent consisted of solution A (Acetonitrile/ Water/phosphoric acid, 70:26:4) and solution B (Potassium Dihydrogen Phosphate at pH 3.5). The gradient program was as follows: starting condition, 85% solution A and 15% solution B for 5 min followed by 70% solution A and 30% solution B for 20 min; successively 50% solution A and 50% solution B for 30 min, then 25% solution A and 75% solution B for 35 min and 5% solution A and 95% solution B for 40 min and finally 85% solution A and 15% solution B for 20 min. Analyses were performed at a flow rate of 1 mL/min and the chromatogram was monitored at 287 nm.

Caffeic acid, protocactechuic acid, *p*-coumaric acid, chlorogenic acid, vanillic acid, eriocitrin, gallic acid, apigenin, dydymin, quercetin, hesperidin, neohesperidin, neoeriocitrin, naringin, narirutin, sinensetin, tangeretin, rutin, isoquercetin, genistin, poncirin, luteolin, kaempferol, hesperetin, rhamnetin, were selected as standard. Identification of compounds was performed by comparing their retention time with those of standards and confirmed with characteristic spectra using the photodiode array detector and literature data (Tundis et al., 2016). Calibration curves, detection limits (LOD), quantification limits (LOQ) of analytical method for determination of phytochemicals in *Citrus* × *clementina* samples were reported in Table 1.

Table 1. Calibration curves, detection limits (LOD), quantification limits (LOQ) of analytical method for determination of phytochemicals in *Citrus* × *clementina* samples

STANDARD COMPOUNDS	Specificity		LOD[a]	LOQ[b]	Precision (RDS %)[d]
	Equation	r^2	mg/L	mg/L	Repeatability
Apigenin	y = 13,331x - 54,145	0,9982	0.001	0.012	0.03
Caffeic acid	y = 235,28x + 9,0586	0,9994	0.003	0.010	0.06
Chlorogenic acid	y = 125,72x - 4,4446	0,9994	0.004	0.025	0.04
Didymin	y = 83,401x + 2,1416	0,9999	0.003	0.018	0.02
Eriocitrin	y = 71,885x -0,2384	0,9999	0.001	0.024	0.08
Gallic acid	y = 39,423x -14,999	0,9994	0.001	0.014	0.02
Genistin	y = 182.21x + 9.8894	0.9993	0.004	0.025	0.04
Hesperidin	y = 15,013x + 1,1586	0,9998	0.001	0.012	0.02
Hesperitin	y = 357.18x + 12.434	0.9999	0.002	0.027	0.05
Kaempferol	y = 271.05x – 94.067	0.9999	0.003	0.018	0.02
Luteolin	y = 154,38x + 9,7542	0,9997	0.001	0.012	0.04
Naringin	y = 76,64x + 12,726	0,9993	0.002	0.027	0.05
Narirutin	y = 81,047x + 3,762	0,9993	0.003	0.014	0.05

Neoeriocitrin	y = 79,482x + 5,9704	0,9992	0.002	0.015	0.04
Neohesperidin	y = 143,95x + 0,5859	0.9999	0.003	0.018	0.02
Nobiletin	y = 13,275x + 6,2888	0.9999	0.004	0.017	0.05
p-Coumaric acid	y = 173,56x + 7,4967	0,9994	0.001	0.014	0.02
Poncirin	y = 190,94x - 1,3754	0.9999	0.002	0.022	0.01
Protocatechuic acid	y = 144,54x +4,3734	0,9994	0.004	0.018	0.03
Quercetin	y = 138,33x - 342,07	0,9991	0.002	0.027	0.03
Isoquercetin	y = 134,99x - 354,9	0,9989	0.002	0.018	0.04
Ramnetin	y = 612.55x – 294.06	0.9999	0.001	0.014	0.02
Rutin	y = 85.419x – 240.8	0.9988	0.001	0.012	0.03
Sinensetin	y = 107,07x + 16,575	0,9997	0.001	0.010	0.04
Tangeretin	y = 168x + 3,2604	0,9992	0.002	0.032	0.06
Vanillic acid	y = 134,01x + 43,634	0,9996	0.001	0.014	0.02

[a] Defined as the signal height at a signal/noise ratio S/N = 3.
[b] Defined as the signal height at a signal/noise ratio S/N = 10.
[c] Precision was expressed as the rsd % of N=3 determination

3.7.2 Furanocoumarins determination

The furanocoumarins determination in *C.* × *clementina* leaves extracts was done. Umbelliferone, isopimpinellin, and bergapten were selected as standard. A concentration of 10 mM was used.

C. × *clementina* extracts were dissolved in a MeOH/H_2O (8:2) solution and filtered on 0.45 µM UptiDisc nylon filters (Interchim, Montluçon, France). The HPLC-DAD-UV analysis was performed on a LaChrom Elite device supplied by VWR (Fontenay-sous-Bois, France) with a D-7000 interface, an L-7200 autosampler, L-7100 pump, L-UV detector. 7400 and running on EZChrom Software Elite 3.3. The Phenomenex C18 column (150 mm × 4.6 mm, 5 µm) was used for analysis and thermostated at room temperature.

The solvents consist of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Thirty µL of each sample were injected and the chromatograms were recorded at $\lambda = 280$ nm. The gradient program was as follows: starting condition, 80% A, 20% B; 0-20 min, 80%-40% A; 20-60 min, 10% A; 60-65 min, 0% A; 65-75 min, 80% A. The flow rate has been set at 0.8 mL/min.

The applied method showed good specificity, linearity ($r^2 \geq 0.9905$), repeatability (RSDs < 0.02–0.04%) and intermediate precision (RSDs < 0.03–0.07%). For selected markers this method is validated for concentrations ranging from 1.00 to 8.00mM because recovery values are between tolerance ranges, 97–107% for umbelliferone 84–106% for isopimpinellin and 98–108% for bergapten, which are included in acceptability intervals (75–125%) (Leporini et al., 2020).

Calibration curves, detection limits (LOD), quantification limits (LOQ) of analytical method for determination of phytochemicals in *Citrus* × *clementina* samples were reported in Table 2.

Table 2. Calibration curves, detection limits (LOD), quantification limits (LOQ) of analytical method for determination of phytochemicals in *Citrus* × *clementina* samples.

STANDARD COMPOUNDS	Specificity		LOD[a]	LOQ[b]	Precision (RDS %)[d]
	Equation	r^2	mg/L	mg/L	Repeatability
Umbelliferone	$y = 1204.2x + 90.928$	0.9946	0.001	0.003	0.02
Isopimpinellin	$y = 2657.5x + 987.46$	0.9930	0.06	0.21	0.04
Bergapten	$y = 1270.1x + 99.83$	0.9905	0.02	0.06	0.03

[a] Defined as the signal height at a signal/noise ratio S/N = 3.
[b] Defined as the signal height at a signal/noise ratio S/N = 10.
[c] Precision was expressed as the rsd % of N=3 determination

3.8 Antioxidant activity

Oxidative stress is a normal phenomenon in the body. Under normal conditions, the physiologically important intracellular levels of reactive oxygen species (ROS) are maintained at low levels by various enzyme systems participating in the in vivo redox homeostasis (Rahal et al., 2014). The evaluation of antioxidant activity is context-dependent. In recent years, many different methods have been proposed for the evaluation of antioxidant activity. Most of them are based on the measurement of the relative abilities of antioxidants to scavenge radicals in comparison with the antioxidant potency of a standard antioxidant compound. The *in vitro* antioxidant activities of *C.* × *clementina* leaves extracts and essential oils were evaluated by using ABTS, DPPH, FRAP and β-carotene bleaching assays.

ABTS assay was applied using the methodology previously described by Loizzo et al. (2014). A solution of ABTS radical cation was prepared by mixing 7 mM ABTS solution with 2.45 mM potassium persulphate and stored at room temperature. After 12 h, the solution was diluted with ethanol to an absorbance of 0.70 at 734 nm using a UV-Vis Jenway 6003 spectrophotometer. Dilution of extracts in ethanol were added to 2 mL of diluted $ABTS^+$ solution in order to test the following concentrations from 400 to 1 μg/mL. After 6 min the absorbance was read at 734 nm. The ABTS scavenging ability was calculated as follows:

$$\text{ABTS scavenging activity (\%)} = [(A0 - A)/A0] \times 100$$

where A0 is the absorbance of the control reaction and A is the absorbance in the presence of extract.

DPPH radical scavenging activity determined according to the technique reported by Loizzo et al. (2018). An aliquot of 1.5 mL of 0.25 mM DPPH radical (DPPH·) in ethanol was mixed with 12 μL of samples in order to test concentrations ranging from 1000 to 1 μg/mL. The mixture was vigorously shaken and allowed to reach a steady state at room temperature for 30 min. The bleaching of DPPH was determined at 517 nm with UV-Vis Jenway 6003 spectrophotometer. The DPPH radicals scavenging activity was calculated according to the following equation:

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

Ascorbic acid was used as positive control in both radical scavenging activity assays.

In the β-carotene bleaching test a mixture of linoleic acid, Tween 20 and β-carotene was prepared (Tundis et al., 2016). One mL of β-carotene solution (0.2 mg/mL in chloroform) was added to 20 μL of linoleic acid and 200 μL of 100% Tween 20. After evaporation of solvent and dilution with water, the emulsion (288 μL) was added into the 96-well microplate containing 12 μL of samples in ethanol concentrations ranging from 100 to 2.5 μg/mL. The plate was shaken and placed at 45 °C in a water bath for 30 and 60 min. The absorbance was measured at 470 nm. Propyl gallate was used as a positive control. The antioxidant activity was calculated as follow:

$$AA = \left(\frac{A_0 - A_t}{A_0^* - A_t^*} \times 100 \right) \pm S.D.$$

where A0 and A0* are the absorbance values obtained at the time 0 for samples and control, respectively, while At and At* are the absorbance values obtained after 30 and 60 min of incubation for samples and control, respectively.

For the preparation of FRAP reagent a mixture of 2.5 mL of 10 mM tripyridyltriazine (TPTZ) solution, 40 mM HCl, 2.5 mL of 20 mM FeCl3 and 25 mL of 0.3 M acetate buffer (pH 3.6) was prepared (Leporini et al., 2020). An aliquot of 100 μL of sample at concentration of 2.5 mg/mL in ethanol was mixed with 2.0 mL of FRAP reagent and 900 mL of water the absorption of the reaction mixture was measured at 595 nm after 30 min of incubation at room temperature. Ethanolic solutions of known Fe (II) concentration, in the range of 50–500 μM (FeSO4), were used for obtaining the calibration curve. FRAP value was expressed as mM Fe(II)/g. Butylated hydroxytoluene (BHT) was used as a positive control.

3.9 Carbohydrate hydrolyzing enzymes inhibition study

Modulation of hyperglycaemia is an important tool in the management of the diabetic patient. α-Amylase and α-glucosidase are enzymes involved in carbohydrates digestion and have been recognized as therapeutic targets for modulation of postprandial hyperglycaemia (Tiwari & Rao, 2002).

In α-amylase inhibitory assay, the enzyme solution was prepared by adding 0.0253 g of enzyme in 100 mL of cold water, and the starch solution was prepared by stirring (at 65 °C for 15 min) 0.125 g of potato starch in 25 mL of sodium phosphate buffer (20 mM) and sodium chloride (6.7 mM) (Loizzo et al., 2018). Samples in ethanol at concentrations ranging from 1000 to 25 μg/mL were added to starch solution and left to react with enzyme at room temperature for 5 min. The absorbance was read at 540 nm.

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

In the α-glucosidase inhibitory activity test a maltose solution was prepared by dissolving 12 g of maltose in 300 mL of 50 mM sodium acetate buffer, α-glucosidase (EC 3.2.1.20) solution was prepared by adding 1 mg of enzyme (10 units/mg) in 10 mL of ice-cold distilled water and *O*-dianisidine (DIAN) solution was prepared by dissolving 1 tablet in 25 mL of distilled water (Loizzo et al., 2018). The peroxidase/glucose oxidase (PGO) system-colour reagent solution was obtained by dissolving 1 capsule in 100 mL of ice-cold distilled water. A mixture of 5 μL of sample (at concentrations ranging from 1000 to 25 μg/mL), 250 μL maltose solution and 5 μL enzyme were left to incubate at 37 °C for 30 min. Then, 50 μL of perchloric acid were added and mixture was centrifuged. The supernatant was collected and mixed with 5 μL of DIAN and 300 μL of PGO, and left to incubate at 37 °C for 30 min. The absorbance was read at 500 nm. The percentage of enzyme inhibition (% I) was calculated by using the following equation:

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

The IC_{50} value for each sample, defined as the concentration of sample causing 50% enzyme inhibition was determined from the curves plotted and tabulated.

3.10 Pancreatic Lipase Inhibition Assay

Pancreatic lipase inhibitory activity was determined by 96-well plate method based on the procedure proposed by El-shiekh et al. (2019). 4-Nitrophenyl octanoate (NPC), 5 mM in dimethylsulfoxide solution and an aqueous solution of porcine pancreatic lipase (1 mg/mL), and Tris-HCl buffer (pH 8.5) were prepared. The samples (2.5-40 mg/mL) were added in a well with 6 μL of the enzyme, 6 μL of NPC and 279 μL of buffer. The mixture was incubated at 37 °C for 30 min. The absorbance was measured at 405 nm. Experiments were performed in triplicate. Orlistat was used as a positive control.

3.11 Statistical analysis

All experiments were carried out in triplicate. Data are expressed as means ± standard deviation (S.D.). The concentration giving 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The concentration-response curve was obtained by plotting the percentage inhibition *versus* concentration.

Relative Antioxidant Capacity Index (RACI) is an integrated statistical application to evaluate the antioxidant capacity values generated by different *in vitro* methods (Sun & Tanumihardjo, 2007). Data obtained from ABTS, DPPH, β-carotene, FRAP were used to calculate the RACI value for the extracts by using the following equation:

$$\text{RACI} = (x - \mu)/\sigma$$

where x is the raw data, μ is the mean, and σ is the standard deviation.

Differences within and between groups were evaluated by one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test (α= 0.05) that was used to compare each group with the positive control in biological assays and Tukey's test to determine any significant difference on chemical parameters among investigated samples at different levels: * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Studies of the *Pearson*'s correlation coefficient (r) and linear regression, assessment of repeatability, calculation of average and relative standard deviation were performed using Microsoft Excel 2010 software.

Principal Component Analysis (PCA) was applied by SPSS software for Windows, version 15.0 (Chicago, IL, USA). Statistical analyses were performed using SPSS software for Windows (SPSS Inc., Elgin, IL, U.S.A.) 22.0 Version and Tukey's test to determine any significant difference among all treatments at $p < 0.05$.

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Chapter 4
Results and discussion

4.1 Introduction

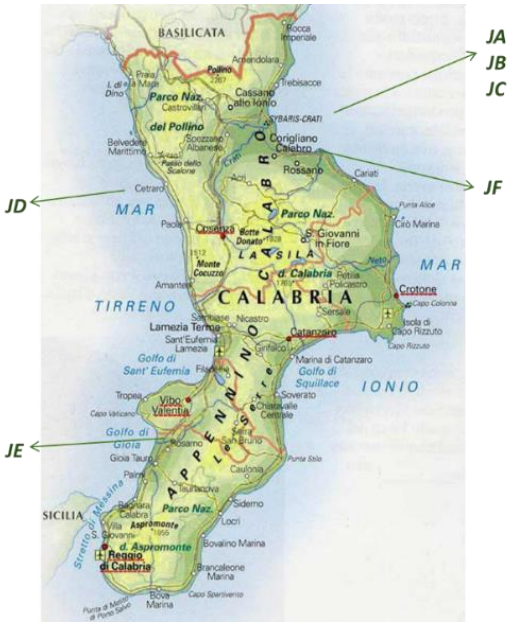
In this project we have investigated the chemical composition and bioactivity of *Citrus* × *clementina* Hort. juice and by-products (leaves and peels) in order to propose their utilization for the development of functional drink useful in prevention and treatment of prediabetes, hyperglycaemia and hyperlipidaemia.

4.2 *C.* × *clementina* samples

The first objective of this project was investigated *C.* × *clementina* juice, obtained from fruits collected in six different areas (Table 1 and Figure 1), for evaluation of their quality and nutritional parameters, chemical profile, antioxidant properties, hypoglycaemic and hypolipidemic potential.

For this purpose, *C.* × *clementina* were collected at fruit maturity stage, defined by visual colour change and size measurement in different areas as reported in Table 2. Fruits were examined for integrity and absence of dust and insect contamination. Fruits were peeled and manually squeezed in order to obtain juice.

Table 1 and Fig. 1. Area of clementine fruits collection



C. × *clementina* juice	JA	JB	JC	JD	JE	JF
Site of collection	Sibari flood plain	Sibari hill	Sibari coastal plain	Cetraro hill	Rosarno hill	Corigliano Calabro Hill

4.2.1 *C.* × *clementina* fruits quality parameters

C. × *clementina* fruit carpometric parameters displayed some statistically significant differences (Table 2). In particular, fruits collected in the flood plain were characterized by a lower weight (87.19 g) and lower fruit firmness (300.56 g/05 cm^2), determined on a portion of peels and 'albedo'.

Table 2. Fruit quality characteristics

Fruit	Fruit weigh (g)	Equatorial diameter (cm)	Longitudinal diameter (cm)	Fruit firmness (g/0.5 cm^2)	Peels thickness (mm)
A	87.19 ± 3.21^{d}	4.71 ± 0.72^{e}	4.54 ± 0.81^{c}	300.56 ± 13.98^{f}	11.23 ± 0.51^{cb}
B	109.67 ± 5.90^{c}	4.42 ± 0.80^{c}	6.23 ± 0.85^{b}	402.12± 12.56^{e}	11.25 ± 0.27^{b}
C	146.61 ± 9.10^{a}	4.93 ± 0.79^{a}	6.72 ± 0.98^{a}	423.92± 14.31^{a}	12.29 ± 0.40^{a}
D	87.19 ± 3.21^{d}	4.67 ± 0.76^{c}	4.54 ± 0.81^{c}	410.19± 12.99^{d}	11.12 ± 0.28^{d}
E	109.67 ± 5.91^{c}	4.83 ± 0.79^{b}	6.23 ± 0.85^{b}	412.28± 14.56^{c}	11.22 ± 0.27^{c}
F	116.61 ± 9.13^{b}	4.55 ± 0.74^{d}	6.72 ± 0.98^{a}	420.92± 14.21^{b}	12.31 ± 0.40^{a}
Sign.	**	**	**	**	**

Data are expressed as mean ± standard deviation (SD) (n= 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same column with different small letters differ significantly ($p< 0.05$).

4.2.2 *C.* × *clementina* juice quality characteristics

Despite the weight differences among the fruits, the difference in percentage yield of juice is minimal with percentage of 45.61 *vs* 49.60% for J2 and J3, respectively.

Table 3. Juice quality parameters

Sample	Juice (%)	Juice (pH)	Acidity (g/100 mL)	°Brix	Croma value (C*)	Hue angle in degrees	Hue angle in radians
JA	47.32 ± 5.07^{c}	3.67 ± 0.07^{b}	0.53 ± 0.03^{e}	10.90 ± 0.02^{b}	31.61 ± 1.11^{b}	71.73 ± 2.14^{e}	1.25 ± 0.05^{f}
JB	45.61 ± 6.40^{d}	3.50 ± 0.05^{c}	0.35 ± 0.05^{f}	9.30 ± 0.01^{f}	30.53 ± 1.12^{d}	72.64 ± 2.13^{d}	1.27 ± 0.09^{e}
JC	49.60 ± 8.42^{a}	3.76 ± 0.08^{a}	0.88 ± 0.02^{a}	11.63 ± 0.02^{a}	30.84 ± 1.10^{c}	72.39 ± 2.14^{d}	1.26 ± 0.08^{d}
JD	48.19 ± 6.07^{b}	3.54 ± 0.06^{c}	0.57 ± 0.03^{d}	10.10 ± 0.02^{e}	30.48 ± 1.12^{d}	73.83 ± 2.02^{c}	1.29 ± 0.09^{c}
JE	47.13 ± 6.01^{c}	3.47 ± 0.05^{c}	0.63 ± 0.05^{c}	10.41 ± 0.03^{c}	28.22 ± 1.09^{e}	75.03 ± 2.12^{b}	1.31 ± 0.10^{b}
JF	48.37 ± 6.42^{b}	3.72 ± 0.08^{b}	0.70 ± 0.03^{d}	10.24 ± 0.02^{d}	34.04± 1.14^{a}	76.06 ± 2.16^{a}	1.33 ± 0.10^{a}
Sign.	**	**	**	**	**	**	**

Data are expressed as mean ± standard deviation (SD) (n= 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same column with different small letters differ significantly ($p< 0.05$). Sign.: significant

The *C.* × *clementina* juice quality parameters (Table 3) evaluated in this study including pH, total soluble solids (TSS), total acidity (TA), and colour were investigated (Figure 2). The values of TA ranged from 0.35 to 0.88 g citric acid/100 mL for JB and JC, respectively. The highest TSS were detected with juice obtained from fruits collected in Sibari, coastal plain (11.63 °Brix). A pH ranging from 3.50 to 3.76 was measured. Significant differences were evidenced in chroma value (C*) where JF presented the highest value (34.04).

Al-Mouei & Choumane (2016) determined the quality parameters of twelve *Citrus* varieties namely common mandarin, mandalina, clementine, Nova, Carvalhal, Dancy, Klimntard, Fortune, Ortanique, Minneola, Ponkan and Satsuma growing in Syria. The TSS in mandarin group ranging from 9.5 to 13.9 °Brix were found.

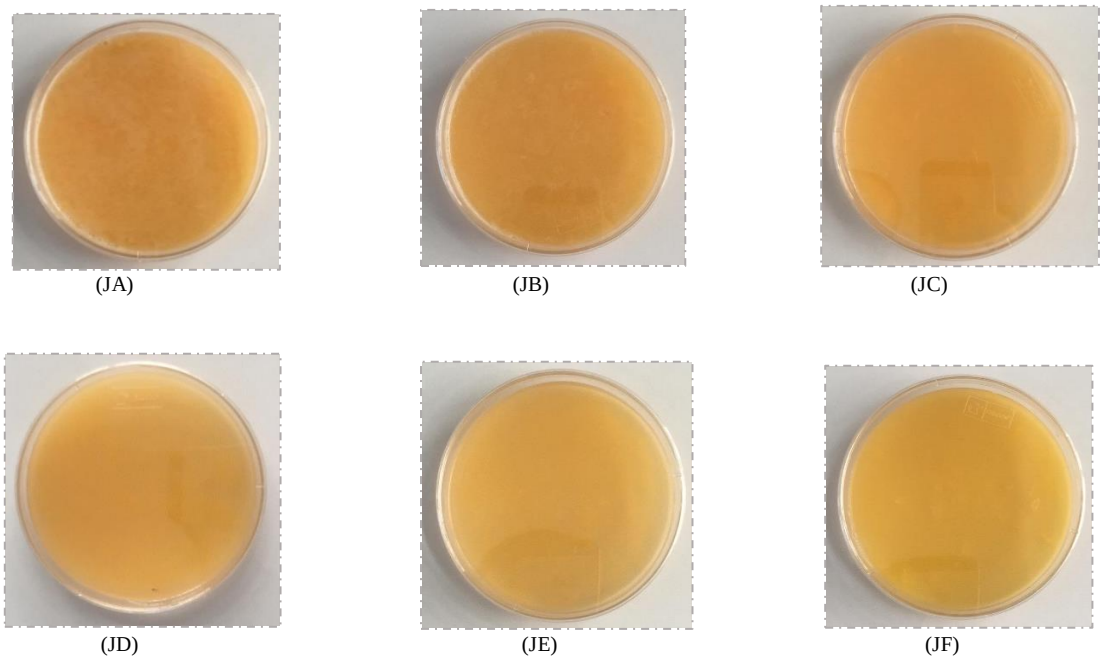


Fig. 2. *C.* × *clementina* juice color.

Our results on pH measurement was in line with those reported by the authors that found a pH value of 3.5 for Clementine while Fortune variety showed the lowest value 2.67. Great variability on total acidity was found also for mandarin group with Minneola that contained high TA value (1.62 g citric acid/100 mL) while Nova has lowest acidity value (0.49 g citric acid/100 mL). Juices from Cadoux, Monreal, St. Martin, Merme, Cheylard, and Rocamora clementine cultivar and one mandarin fruit cultivar were investigated for quality parameters and antioxidant potential (Boudries et al., 2012). The TA of investigated juices ranged from 4.61 to 7.47 g of citric acid/L for clementine cv. Merme and mandarin, respectively, with a pH varied between 3.91 and 3.68 for the same cultivars, respectively. Fruit juices of Safor [(*C. clementina* × *C. tangerina*) × (*C. unshiu* × *C. nobilis*)], Garbí [(*C. clementina* × *C. tangerina*) × (*C. reticulata* × *C. sinensis*)], Fortune (*C. clementina* × *C. tangerina*), Kara (*C. unshiu* × *C. nobilis*) and Murcott (*C. reticulate* × *C. sinensis*) were investigated for juice quality parameters including juice yield, TA and TSS (Sdiri et al., 2012).

A percentage of juice ranging from 43.3 to 59.9% was found for Murcott and Fortune, respectively. Garbí juice displayed the greatest total soluble solids values whereas Murcott presented the lowest values. With respect to the acidity, all values were higher than those found for Calabria clementine juice with similar total acidity values for Garbí, Fortune and Kara juice. Similar values of juice yield, TSS and TA were evidenced also in Wase-Satsuma, Satsuma, Ponkan, Bendizao, Manju, new variety Hybrid 439 and Zhuhong mandarins from China (Xu et al., 2008). Manju variety achieved the highest yield (60.74%) while Hybrid 439 had the highest TSS value (14.92%).

The juice of thirteen cultivars of *C. clementina* was investigated for its quality parameters and radical scavenging potential (Milella et al., 2011). A pH values ranging

from 2.60 to 3.58 Mandalate and Spinoso cultivars, respectively, were found. In line with our results, samples showed a total acidity ranging from 9.25 to 12.12 °Brix for Rubino and RA92 cultivars, respectively.

Evolution of juice yield, TSS, and TA of new pigmented *Citrus* hybrid namely Omo-31 and those of its parent clementine cv. Oroval (*C. clementina*) and Moro orange (*C. sinensis*) were investigated during fruit maturation (Rapisarda et al., 2003). Results clearly evidenced that juice yield, TSS and TA values of new pigmented *Citrus* hybrid were similar to those of the Moro orange. The juice obtained from fruits collected in Sibari coastal plain was richest in ascorbic acid (66.25 mg/L). No significant differences in TPC and TFC content were found, with TPC content ranging from 29.74 to 44.20 mg CAE/100 mL for JA and JC, respectively and a TFC content from 16.48 to18.23 mg QE/100 mL for JA and JF, respectively. Differently, the TCC was influenced by fruits area of collection. In particular, TCC content in JB was 1.5 times higher than JC. All samples showed a low-fat content, a fiber content between 1.76 and 186 for JB and JC respectively, and a similar carbohydrates content (12.01-12.35, for JB and JC respectively). Mineral analysis showed that all investigated cultivars are rich in potassium.

Our values disagree with those reported by Casacchia et al. (2019) that found a TPC value of 90.01 mg CAE/g FW and a TFC of 41.3 mg QE/g FW in *C.* × *clementina* pulp, collected in Mirto-Crosia.

Lower values of vitamin C were detected by Bermejo et al. (2016) that screened 15 mandarin cultivars. Among them, Arrufatina, Loretina and Fina displayed the highest values of total vitamin C, followed by the hybrid Ellendale. Previously, Al-Mouei & Choumane (2014) determined the quality parameters of twelve *Citrus* varieties namely common mandarin, mandalina, clementine, Nova, Carvalhal, Dancy, Klimntard, Fortune, Ortanique, Minneola, Ponkan and Satsuma growing in Syria. Among all investigated varieties group, Ortanique had the highest juice content (56.1%) while common mandarin had the lowest content (37%). Ascorbic acid content was lowest in clementine St Martin cv. (35.98 mg/100 mL). Ascorbic acid content of Hybrid 439 agrees with those reported for our samples (Milella et al., 2011). A great variability was found in ascorbic acid content with values from 205.85 to 643.73 mg/L for Mandalate and Fedele cultivars, respectively. The high content of healthy compounds was confirmed also in this study since authors reported a mean content of β-carotene of 13.58 mg/L. Similar results, were also obtained by RA 85 and in RA 133 cultivars by Dhuique-Mayer et al. (2005) and in two new mandarin-like hybrids (*C. clementina* × *C. sinensis*) by Rapisarda et al. (2008). No differences were observed for *Citrus* hybrid namely Omo-31 and those of its parent clementine cv. Oroval (*C. clementina*) and Moro orange (*C. sinensis*) on vitamin C content at maturity stage (47–48 mg/100 mL of juice).

Table 4. *C.* × *clementina* juice content and nutritional constituents

Parameter	JA	JB	JC	JD	JE	JF	Sign.
Ascorbic acid (mg/100 mL)	65.92 ± 3.46^{a}	60.40 ± 3.26^{b}	66.25 ± 3.88^{a}	65.12 ± 3.23^{a}	64.48 ± 3.26^{a}	66.34 ± 3.88^{a}	ns
TPC (mg CAE/100 mL)	29.74 ± 1.12^{d}	32.16 ± 1.18^{b}	44.20 ± 1.28^{a}	29.46 ± 1.11^{d}	30.28 ± 1.17^{dc}	31.12 ± 1.19^{b}	**
TFC (mg QE/100 mL)	16.48 ± 0.90^{c}	17.22 ± 0.92^{cb}	17.65 ± 0.95^{d}	17.58 ± 0.93^{cb}	18.16 ± 0.99^{a}	18.23 ± 0.92^{a}	**
TCC (mg β-CE/100 mL)	42.89 ± 2.83^{e}	75.45 ± 2.79^{a}	49.69 ± 2.56^{d}	54.65 ± 2.92^{b}	51.48 ± 2.84^{c}	53.54 ± 2.89^{cb}	**
Nutritional constituents (g/100 g)							
Ash	0.43 ± 0.04^{b}	0.44 ± 0.03^{b}	0.47 ± 0.05^{a}	0.45 ± 0.04^{b}	0.43 ± 0.03^{b}	0.47 ± 0.05^{a}	**
Fat	0.15 ± 0.09^{ab}	0.15 ± 0.09^{b}	0.16 ± 0.10^{ab}	0.17 ± 0.11^{a}	0.16 ± 0.19^{ab}	0.15 ± 0.10^{ab}	**
Protein	0.85 ± 0.09^{a}	0.88 ± 0.08^{a}	0.89 ± 0.07^{a}	0.80 ± 0.09^{a}	0.83 ± 0.07^{a}	0.82 ± 0.08^{a}	ns
Fiber	1.78 ± 1.10^{cd}	1.76 ± 1.01^{d}	1.86 ± 1.13^{a}	1.82 ± 1.12^{b}	1.76 ± 1.04^{d}	1.80 ± 1.14^{cb}	**
Carbohydrates	12.14 ± 1.86^{a}	12.02 ± 1.85^{a}	12.35 ± 1.87^{a}	12.31 ± 1.84^{a}	12.10 ± 1.80^{a}	12.01 ± 1.82^{a}	ns
Energy kcal/100 g	53 ± 2.02^{a}	53 ± 2.03^{a}	54 ± 2.04^{a}	54 ± 2.03^{a}	53 ± 2.04^{a}	52 ± 2.02^{a}	ns
Minerals (mg/100 g)							
Phosphorus	19 ± 1.31^{b}	21 ± 1.58^{a}	22 ± 1.46^{a}	20 ± 1.21^{a}	21 ± 1.33^{a}	22 ± 1.41^{a}	**
Potassium	177 ± 4.46^{a}	179 ± 4.47^{a}	185 ± 4.56^{a}	181 ± 4.52^{a}	178 ± 3.33^{a}	183 ± 3.56^{a}	ns
Calcium	30 ± 1.28^{a}	32 ± 1.29^{a}	32 ± 1.28^{a}	31 ± 1.24^{a}	30 ± 1.27^{a}	31 ± 1.25^{a}	ns
Magnesium	14 ± 0.90^{a}	10 ± 0.80^{c}	14 ± 0.92^{a}	12 ± 0.83^{b}	12 ± 0.91^{b}	13 ± 0.94^{a}	**

TPC: Total Phenols Content (mg equivalents of chlorogenic acid/100 mL); TFC: Total Flavonoids Content (mg equivalents of quercetin/100 mL); TCC: Total Carotenoids Content (mg equivalents of β-carotene/100 mL). Data are expressed as mean ± standard deviation (SD) (n= 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same row with different small letters differ significantly (p< 0.05). ns: not significant.

4.2.3 HPLC–DAD analysis of *C.* × *clementina* juice

Phenolic determination

HPLC-DAD phenolic profile evidenced the presence of nineteen flavonoids namely eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, didymin, neohesperidin, poncirin, quercetin, apigenin, sinensetin, nobiletin, and tangeretin selected as markers. Data are reported in Table 5.

Among identified constituents, the flavanone glycoside neohesperidin was the main abundant compound (72.96-116.50 mg/100 mL for JA and JC, respectively) followed by the flavanone aglycones hesperidin (40.06–81.08 mg/100 mL for JD and JA, respectively). However, several differences were displayed. In fact, neohesperidin was 1.6-times higher in JA in comparison to JC. The same trend was observed for hesperidin in fact JD had a double content respect JE. Significant amounts of narirutin (6.25–12.13 mg/100 mL) were also detected. The flavanone-*O*-glycosides didymin was mainly contained in JA while, eriocitrin in JE. Interestingly, naringin was not detected in JB. Chlorogenic acid, vanillic acid, caffeic acid and gallic acid were also quantified. Caffeic acid was the main abundant compound with particular reference to JF (7.48 mg/mL) followed by chlorogenic acid (2.06-3.59 mg/mL).

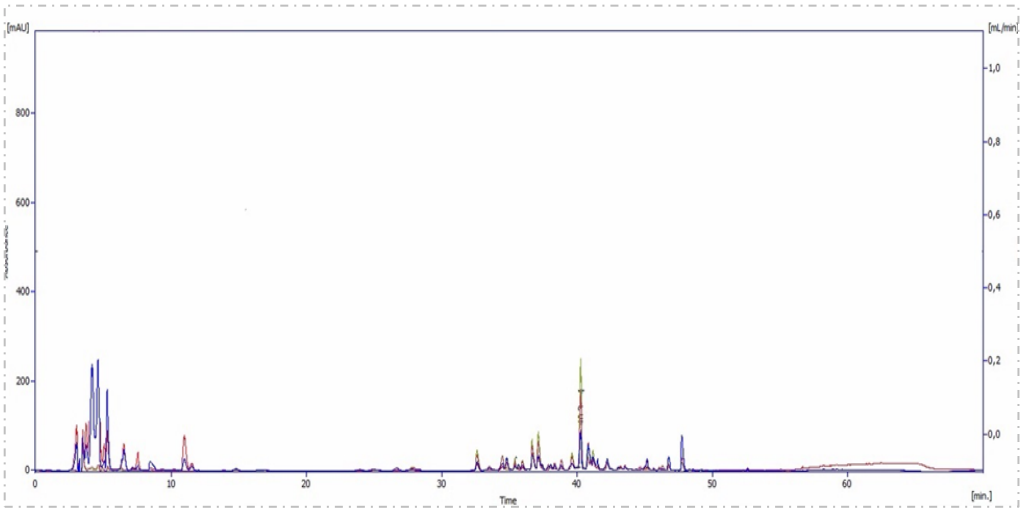


Fig. 3. JF phenolic profile

The trend hesperidin > narirutin > didymin was confirmed also by Bermejo et al. (2016) for all investigated samples except Murcott and Murta cultivars. Gattuso et al. (2007) reported the chemical composition of *C.*× *clementina* juice composition in which high content hesperidin (9.9 mg/100 mL), followed by narirutin (4.64 mg/100 mL) were found.

A similar trend was observed also by Rapisarda et al. (2003) for clementine collected in Acireale, Sicily. In the same study, the juice of hybrid Omo-narirutin was not the second abundant flavanone glycoside after hesperidin. Higher values were reported by Milella et al. (2011) that found a hesperidin content ranging from 63.98 to 165.88 mg/L for Etna hybrid and Rubino cultivars, respectively.

Table 5. HPLC analysis of selected markers of *C.* × *clementina* juice. Results are expressed as mg/100 mL

Selected markers	JA	JB	JC	JD	JE	JF	Sign.
Chlorogenic acid	3.59 ± 1.45^{a}	2.88 ± 1.30^{b}	2.07 ± 1.27^{d}	2.59 ± 1.22^{c}	2.06 ± 1.20^{d}	2.56 ± 1.26^{c}	**
Protocatechuic acid	0.95 ± 0.07^{d}	1.03 ± 0.81^{c}	1.13 ± 0.93^{b}	1.02 ± 0.74^{c}	0.62 ± 0.07^{e}	1.67 ± 0.96^{a}	**
Caffeic acid	6.23 ± 1.50^{d}	5.24 ± 1.41^{e}	6.78 ± 1.59^{c}	7.48 ± 1.61^{b}	3.65 ± 1.30^{f}	8.52 ± 1.81^{a}	**
Vanillic acid	1.87 ± 0.10^{c}	1.91 ± 0.19^{b}	0.44 ± 0.05^{e}	0.69 ± 0.07^{d}	2.16 ± 1.30^{b}	0.43 ± 0.03^{e}	**
p-Coumaric	5.61 ± 1.50^{e}	6.74 ± 1.63^{c}	5.27 ± 1.42^{f}	7.67 ± 1.69^{b}	6.47 ± 1.58^{d}	8.29 ± 1.72^{a}	**
Apigenin	0.09 ± 0.07^{a}	0.05 ± 0.03^{d}	0.08 ± 0.04^{b}	0.06 ± 0.04^{d}	0.05 ± 0.03^{c}	0.09 ± 0.08^{a}	**
Didymin	5.65 ± 1.54^{a}	3.65 ± 1.24^{f}	5.36 ± 1.35^{c}	3.85 ± 1.26^{e}	4.17 ± 1.33^{d}	5.51 ± 0.04^{b}	**
Eriocitrin	1.27 ± 0.12^{d}	1.14 ± 0.11^{f}	1.19 ± 0.14^{e}	1.84 ± 0.19^{c}	2.33 ± 1.34^{a}	0.91 ± 0.11^{b}	**
Gallic acid	1.25 ± 0.13^{c}	1.77 ± 0.15^{a}	0.85 ± 0.06^{e}	1.02 ± 0.09^{d}	0.62 ± 0.04^{f}	1.67 ± 0.18^{b}	**
Hesperidin	55.24 ± 2.53^{e}	60.39 ± 4.21^{d}	69.52 ± 3.88^{b}	81.08 ± 4.94^{a}	40.06 ± 3.04^{f}	65.3 ± 3.54^{c}	**
Naringin	1.97 ± 0.19^{c}	*n.d*	1.14 ± 0.08^{e}	1.73 ± 0.18^{d}	2.12 ± 1.19^{b}	3.14 ± 1.32^{a}	**
Narirutin	9.91 ± 1.94^{b}	12.13 ± 1.92^{a}	7.21 ± 1.70^{e}	8.50 ± 1.83^{c}	6.25 ± 1.32^{f}	7.88 ± 1.81^{d}	**
Neoeriocitrin	2.25 ± 0.54^{f}	3.5 ± 1.08^{a}	3.2 ± 1.07^{c}	2.69 ± 0.72^{e}	3.14 ± 1.04^{d}	3.41 ± 1.39^{b}	**
Neohesperidin	116.50 ± 5.63^{a}	107.47 ± 5.29^{d}	72.96 ± 4.49^{f}	110.63 ± 5.52^{c}	80.26 ± 4.83^{e}	112.32 ± 5.63^{a}	**
Nobiletin	0.10 ± 0.01^{a}	0.09 ± 0.01^{c}	0.15 ± 0.01^{d}	0.06 ± 0.01^{a}	0.12 ± 0.01^{b}	0.14 ± 0.01^{a}	**
Poncirin	2.15 ± 0.51^{b}	1.28 ± 0.12^{f}	1.88 ± 0.23^{c}	1.52 ± 0.19^{e}	1.74 ± 0.21^{d}	2.63 ± 0.51^{a}	**
Quercetin	0.60 ± 0.04^{a}	0.26 ± 0.02^{c}	0.25 ± 0.02^{c}	0.42 ± 0.03^{b}	0.56 ± 0.05^{c}	1.32 ± 0.13^{a}	**
Sinensetin	0.006 ± 0.01^{b}	0.008 ± 0.01^{ab}	0.008 ± 0.01^{ab}	0.005 ± 0.01^{b}	0.006 ± 0.01^{b}	0.01 ± 0.03^{a}	**
Tangeretin	0.05 ± 0.04^{b}	0.06 ± 0.07^{ab}	0.05 ± 0.06^{b}	0.06 ± 0.07^{ab}	0.05 ± 0.06^{b}	0.07 ± 0.08^{a}	**
∑ Indentified Phenolics	215.86	209.598	186.54	232.92	156.44	226.01	

Data are expressed as mean ± standard deviation (SD) (n= 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same row with different small letters differ significantly (p< 0.05). Sign: significant.

Previously Xu et al. (2008) quantified narirutin, hesperidin, naringin, and neohesperidin in different *Citrus* varieties (Wase-Satsuma, Satsuma, Ponkan, Bendizao, Manju, new variety Hybrid 439 and Zhuhong). Interestingly, in spite of our data in which the flavanone glycoside neohesperidin represents the main abundant compound in *Citrus* juice from China is not detected together with naringin. On the contrary, Nogata et al. (2006) reported the presence of rutin in significant amount in *C.× clementina* juice

Coumarins determination

In order to exclude the presence of the most common coumarins (umbelliferone, isopimpinellin and bergapten) in *C. × clementina* juice, HPLC-UV analysis in all juice samples was performed. The obtained data excluded the presence of furanocoumarins in all investigated samples.

Furanocoumarins have controversial effects on humans both acting as potential photosensitizers and interacting with drugs with inhibition of the intestinal cytochrome P450-3A4 (Dugrand et al., 2013; Lin et al., 2012). For patients undergoing drug therapy, the inhibition of cytochrome P450-3A4 by furanocoumarins may lead to a higher concentration of drug in the blood, which in turn can cause serious side effects such as heart rhythm disturbances or respiratory depression (Bailey et al., 2013). Removing furanocoumarins from food implies additional costs and might alter product quality (Costa et al., 2019). Thus, the selection of *Citrus* cultivars displaying low furanocoumarin contents constitutes a valuable alternative for their industrial use.

The applied method showed good specificity, linearity ($r^2 \geq 0.9905$), repeatability (RSDs < 0.02-0.04%) and intermediate precision (RSDs < 0.03-0.07%). For selected markers this method was validated for concentrations ranging from 1.00 to 8.00 mM. Recovery values were between tolerance ranges, 97-107% for umbelliferone 84-106% for isopimpinellin and 98-108% for bergapten, which were included in acceptability intervals (75-125).

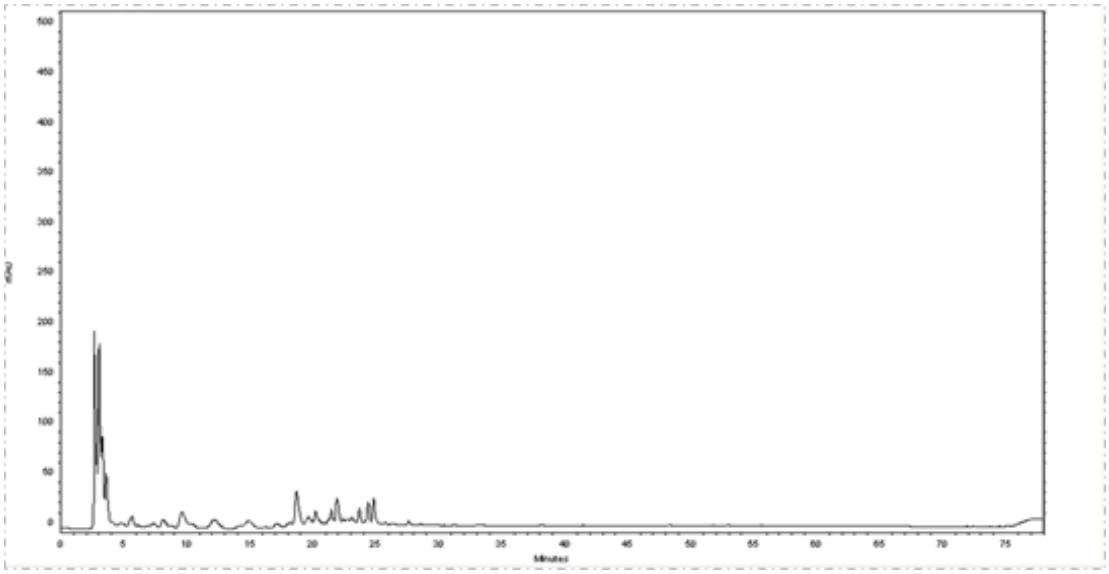


Fig 4. Cumarins determination in JF

Dugrand-Judek et al. (2015) reported that environmental factors, such as the exposure to air and water pollution, stimulated furanocoumarin biosynthesis. Additionally, the presence of these secondary metabolites was influence by phenotypic diversity and the intraspecific chemo-diversity of *Citrus* species and suggested that plants related to *C. maxima*, *C. micrantha*, *C. lemon*, *C. hystrix*, accumulated these compounds in high amounts. While, *C. deliciosa* and related species appeared almost devoid of them. Concerning hybrids, their corresponding chemotypes appeared inherited from respective ancestral taxa, with a prevalence of *C. maxima*, *C. lemon*, and C. *hystrix* related species and hybrids.

4.2.4 Antioxidant activity of *C.* × *clementina* juice

An antioxidant is defined as a molecule capable to reduce or inhibit the oxidation of other molecules, whereas a biological antioxidant has defined as "any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or prevents oxidation of substrate".

Recently, polyphenols/flavonoids found in plants get much attention among researchers as a new natural antioxidant. Several methods were developed for measuring the total antioxidant capacity of a matrix; these assays differ in their chemistry such as generation of different radicals and/or target molecules. Different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the antioxidant activity of a matrix. For this reason, in this project the antioxidant properties of samples were investigated using different methods: ABTS, DPPH, FRAP, and *β*-carotene bleaching tests.

The radical scavenging activity of juice samples were examined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and the 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals have a different stereochemistry and a different training mechanism and therefore, after reaction with antioxidants, they give a qualitatively different response to the inactivation of their radical (Loizzo et al., 2018). The potential of *C.* × *clementina* juice to inhibit lipid peroxidation was assessed using the β-carotene bleaching test. The ability of sample to induce the reduction of TPTZ-Fe^{3+} was measured with FRAP test. Data are reported in Table 6.

A concentration-dependent activity was observed for all samples independently by the applied methods. Juice obtained from fruits collected in Corigliano Calabro exhibited the highest radical potential with IC_{50} values of 81.13 and 27.82 μg/mL for DPPH and ABTS test, respectively followed by the JD (IC_{50} of 82.43 and 33.63 μg/mL for DPP; and ABTS, respectively). Moreover, in β-carotene bleaching test JF presented the highest protection of lipid peroxidation with percentage of 31.33 and 34.20%, respectively after 30 and 60 minutes of incubation. In FRAP assay, the activity of the juices was minimal, JA and JB were inactive, while JF showed an IC_{50} value of 5.70 μM Fe (II)/g.

Table 6. Antioxidant activity of *C.* × *clementina* juice

Sample	**DPPH test**	**ABTS test**	**β-carotene bleaching test**	**β-carotene bleaching test**	**FRAP test**
	IC_{50} (µg/mL)	IC_{50} (µg/mL)	**t 30 min[a]**	**t 60 min[a]**	µM Fe (II)/g
JA	97.14 ± 3.61****	47.92 ± 2.47****	15.06 %	17.70 %	*na*
JB	108.32 ± 4.14****	65.21 ± 3.69****	19.69 %	20.42 %	*na*
JC	88.97± 3.18****	56.11 ± 2.82****	10.24 %	11.67 %	1.74 ± 0.06****
JD	82.43 ± 2.96****	33.63 ± 2.01****	25.90 %	27.50 %	2.8 ± 0.96****
JE	84.02 ± 2.92****	40.32 ± 2.54****	27.50 %	28.47 %	3.01 ± 0.98****
JF	81.13 ± 2.73****	24.82 ± 1.96****	31.33 %	34.20 %	5.70 ± 1.00****
Positive control					
Ascorbic acid	5.01 ± 0.80	1.72 ± 0.06			
Propyl gallate			0.09 ± 0.004	0.09 ± 0.004	
BHT					63.23 ± 4.31

Data are expressed as means ± S.D. (n= 3). DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$); β-carotene bleaching test; Ferric Reducing Antioxidant Power (FRAP). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. [a]Juice as it is. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the positive controls. *na*: not active.

Pearson's correlation coefficient was reported in Table 7. In particular, the most significant correlations were found between the content in narirutin and DPPH with r = 0.87. Also, a positive correlation between naringenin, eriocitrin and *p*-coumaric acid and β-carotene bleaching test was found. In addition, the *Pearson*'s correlation coefficient was positive between caffeic acid, protocatechuic acid and tangeretin and FRAP with r = 0.80, 0.81, and 0.79 respectively.

Table 7. Positive *Pearson*'s correlation between phenolic compounds content in investigated samples and activity

	DPPH test	ABTS test	β-carotene bleaching test t 30 min	β-carotene bleaching test t 60 min	FRAP test
TPC	0.21	0.55			
TFC			0.72	0.62	0.67
TCC	0.57	0.45			
Ascorbic acid			0.48	0.42	0.69
Caffeic acid			0.51	0.49	0.81
Protocatechuic acid			0.49	0.44	0.80
p-Coumaric acid			0.75	0.82	0.73
Chlorogenic acid	0.54	0.15			
Vanillic acid	0.55	0.41			
Eriocitrin			0.78	0.79	0.47
Gallic acid	0.50	0.09	0.08	0.13	0.26
Apigenin			0.31	0.27	0.33
Didymin		0.13	0.20	0.13	0.31
Quercetin			0.39	0.46	0.42
Hesperidin					0.34
Neohesperidin	0.21		0.18	0.32	0.20
Neoeritrocin	0.18	0.24	0.15	0.04	0.26
Naringin			0.87	0.86	0.73
Narirutin	0.87			0.86	0.73
Poncirin			0.66	0.60	0.67
Sinensetin					0.56
Tangeretin			0.62	0.64	0.79

The RACI values of each juice were calculated as the mean of standard scores transformed from the raw data generated with different antioxidant methods. The difference in units and variances in the raw data had no influence on the RACI. Stepwise regression between RACI and different chemical methods revealed that (a) each of the assays was selected as a significant variable with no single applied method being removed, (b) each method contributed the same weight in building RACI, and (c) the regression was highly significant (r = 1, $p < 0.001$). Therefore, RACI of each juice is a scientific combination of data from different antioxidant methods with no unit limitation and no variance among methods, and makes comparison of matrix antioxidant capacity probable and possibly more accurate. Based on RACI data, the following antioxidant rank order has been found: JF > JD > JE (Figure 5). This trend clearly evidenced that JF had the highest antioxidant potential.

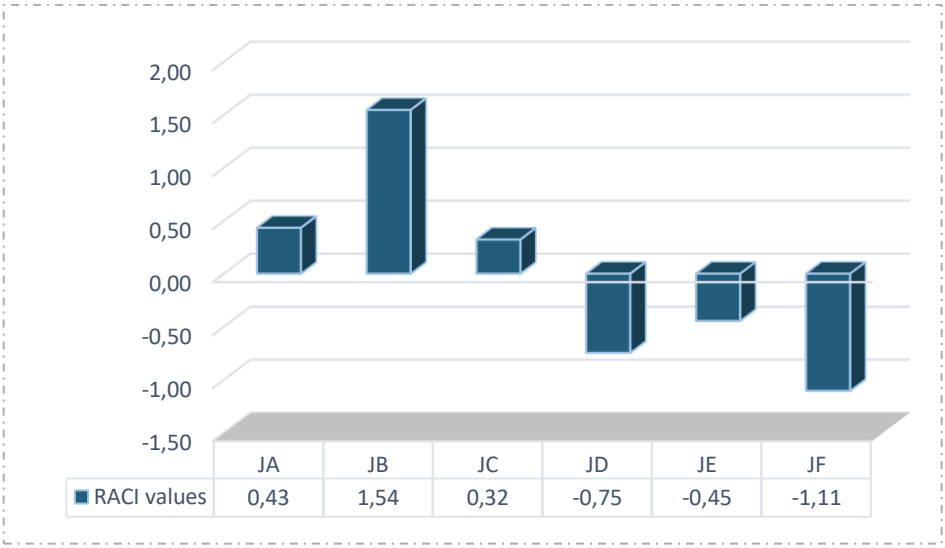


Fig. 5. RACI values of *C.* × *clementina* juices

Previously, Boudries et al. (2012) reported the strong radical scavenging potential of clementine with IC_{50} values from 1.14 to 1.91 mg/mL for Merme and St Martin cultivars, respectively. No significative differences in DPPH· radical scavenging ability were found in Safor, Fortune, Kara, Murcott juice with the only exception of Garbí juice. This evidence is probably due to the lower level of ascorbic acid in Garbí mandarin (21.19 mg/100 mL) (Sdri et al., 2012). A great variability in radical scavenging potential was found by Xu et al. (2008) that showed a percentage of inhibition of DPPH· radical from 23.69 to 61.62% for Manju and hybrid 439, respectively, at maximum concentration tested. The radical scavenging potential of clementine mandarins was confirmed also by Russo et al. (2012) that found as Caffin, Fedele, Ragheb and RA89 juice methanol extracts from fruits collected in Metaponto (Basilicata region, Italy) showed the highest ABTS radical scavenging activity with values from 23.77 to 25.52 mg Trolox equivalent/100 mL of juice, respectively.

Several identified compounds can scavenge DPPH radical. In particular, the main abundant constituent neohesperidin showed in DPPH test an IC_{50} value of 13.40 mM. Values of 16.54, 36.16 and 45.30 mM were recently reported for hesperidin, didymin and narirutin by Tundis et al. (2016a).

The antioxidant effect of *Citrus mandarin* varieties was confirmed also trough *in vivo* study by Codoñer-Franch et al. (2008). Diet supplementation of hypercholesterolemic children with 500 mL/day of pure (100%) mandarin juice (*C.* × *clementina*) for 28 days results in a strong reduction of plasma biomarkers levels of oxidative stress, whereas the plasma antioxidants vitamin E and C and intraerythrocyte glutathione level were significantly increased.

4.2.5 Hypoglycaemic and hypolipidemic activity of *C.* × *clementina* juice

Several research articles evidenced that oxidative stress, obesity and T2DM are strictly related (Xu et al., 2018).

Table 8. Hypoglycaemic and hypolipidemic activity of *C.× clementina* juice

Sample	**α-Amylase** IC_{50} (μg/mL)	**α-Glucosidase** IC_{50} (μg/mL)	**Lipase** IC_{50} (μg/mL)
JA	238.86 ± 2.89****	200.92 ± 2.68****	241.37 ± 2.98****
JB	226.60 ± 2.78****	77.79 ± 1.74****	210.51 ± 2.75****
JC	243.24 ± 2.38****	93.31 ± 2.16****	212.63 ± 2.64****
JD	189.81± 2.09****	89.37± 2.07****	192.14 ± 2.47****
JE	194.33± 2.15****	103.43± 2.43****	197.69 ± 2.68****
JF	139.89± 1.81****	67.19 ± 1.31****	179.32 ± 2.19****
Positive control			
Acarbose	50.01 ± 0.92	35.52 ± 1.23	
Orlistat			37.63 ± 1.01

Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Orlistat use as positive control in lipase test. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the positive control.

For this reason, *C.* × *clementina* juice was tested for its potential inhibitory activity against α-enzymes, α-glucosidase and lipase enzymes. The inhibition of carbohydrate hydrolysing enzymes α-amylase and α-glucosidase was investigated and results are reported in Table 8.

All investigated samples inhibited both enzymes in a concentration-dependent manner. Generally, the most promising activity was found against α-glucosidase. In particular, JF exhibited the highest inhibitory activity with IC_{50} value of 67.19 μg/mL, followed by JA with IC_{50} value of 77.79 μg/mL ($p < 0.0001$, $\alpha = 0.05$). However, several differences were evidenced against α-amylase. In fact, JF (IC_{50}= 139.89 μg/mL) was 1.7-times higher active in comparison to JC (IC_{50}= 243.24 μg/mL).

Pearson's correlation coefficient revealed a positive correlation between chlorogenic acid and α-glucosidase with *r* value of 0.69, while TPC positively correlated with α-amylase (Table 9).

Table 9. Positive *Pearson*'s correlation between phenolic compounds content in investigated samples and activity

	α-Amylase	α-Glucosidase	Lipase
TPC	0.39		
Ascorbic acid		0.08	
Chlorogenic acid	0.25	0.69	0.63
Vanillic acid	0.36	0.45	0.48
Apigenin		0.48	0.26
Didymin	0.48	0.44	0.29
Quercetin		0.47	0.13
Neohesperidin		0.24	0.06
Naringin		0.09	
Narirutin	0.34	0.15	0.41
Nobiletin	0.19	0.48	0.43
Poncirin		0.17	

Metibemu et al. (2016) demonstrated that when chlorogenic acid target α-amylase a binding energy of -7.3 Kcal/mol was found. This value is slight better than acarbose. An energy value of -5.97 Kcal/mol was found by Pérez-Nájera et al. (2018). The main interactions of chlorogenic acid with α-amylase involved conventional hydrogen bonds with residues LYS^{200}, GLU^{240}, GLY^{306}, and GLY^{308}.

The same research group investigated the interaction between chlorogenic acid and α-glucosidase enzyme. The authors found that the binding site for chlorogenic acid on *α*-glucosidase was situated in a place away from the active site. The complex of *α*-glucosidase-chlorogenic acid showed lowest binding energy of -3.75 kcal/mol. Chlorogenic acid formed hydrogen bonds with the residues SER^{161}, PHE^{165}, and LYS^{418}, and a π-π T-shaped interaction between A ring and PHE^{172} was found. It is likely that

this binding interaction of chlorogenic acid to the allosteric site of α-glucosidase would probably perturb the protein structure and subsequently the enzyme activity.

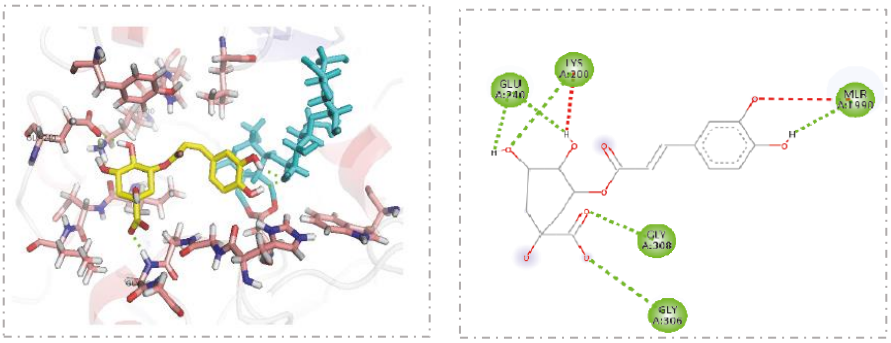


Fig. 6. Molecular docking for chlorogenic acid with α-amylase (Adapted from Metibemu et al., 2016)

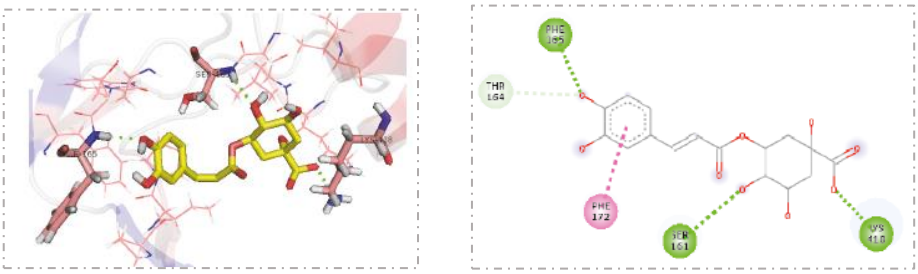


Fig. 7. Molecular docking for chlorogenic acid with α-glucosidase (Adapted from Metibemu et al., 2016)

The efficacy of several *Citrus* juice in the management of diabetes is supported by conclusive evidence from *in vitro* and *in vivo* models. Recently, Tundis et al. (2016a) reported that *Poncirus trifoliata* (L.) Raf. juice inhibited α-amylase and α-glucosidase with IC_{50} values of 138.14 and 81.27 µg/mL, respectively. Fresh juice from fruits *C. hystrix* and *C. maxima* showed *in vitro* hypoglycaemic effect with inhibition of 75.55 and 79.75% against α-amylase, and 70.68 and 72.83% against α-glucosidase, respectively (Abirami et al., 2014). Moreover, *C. paradisi* juice significantly reduced rapid blood glucose levels without any effect on 1.5-h plasma insulin levels (Jim et al., 2016). Previosly, Mollace et al. (2011) demonstrated that bergamot juice extract, administered for 30 days in Wistar rats and in 237 patients affected by hyperlipemia associated or not with hyperglycaemia, is able to induce a significant decrease in blood glucose level in both rats and patients.

Among *Citrus* phytochemicals, flavonoids are mainly involved in the management of T2DM. These compounds are able to *i*) inhibit carbohydrate hydrolysing enzymes (Tundis et al., 2016a); *ii*) inhibit sodium-dependent glucose transporter 1 (SGLT1) (Kim et al., 2016); *iii*) stimulate insulin secretion; *iv*) reduce hepatic glucose output; and *v*) enhance insulin-dependent glucose uptake (Jia et al., 2015). In particular, the main abundant flavonoids of *C.* × *clementina* juice, neohesperidin, inhibited both α-amylase and α-glucosidase in a concentration-dependent manner, and was more active than the

prescribed drug acarbose. Additionally, Jia et al. (2015) demonstrated that neohesperidin, significantly reduced serum glucose and glycosylated serum protein *in vivo*.
Other bioactive compounds are didymin that showed an IC_{50} value of 4.20 μM against α-glucosidase, followed by naringin (IC_{50} value of 10.33 μM), narirutin (IC_{50} value of 14.30 μM), and hesperidin (IC_{50} value of 15.89 μM). This last flavanone glycoside was able to inhibit α-amylase with IC_{50} value of 26.04 μM. Among flavonoids identified in clementine juice the most active against α-amylase was neoeriocitrin with an IC_{50} value of 4.69 μM (Tundis et al., 2016a). Previously, Shen et al. (2012) studied the effect of hesperidin, naringin, neohesperidin, and nobiletin on amylase-catalysed starch digestion, pancreatic α-amylase and α-glucosidase, and glucose utilization. All investigated flavonoids are able to inhibit amylase-catalysed starch digestion. Neohesperidin and naringin principally inhibited amylose digestion, whereas hesperidin, inhibited both amylose and amylopectin digestion. Similar, the inhibitory effects on α-amylase for naringin, neohesperidin, and hesperidin were investigated by Liu et al. (2017). These flavonoids presented different binding ability with the order of naringin > neohesperidin > hesperidin. The antioxidant activity of these *Citrus* flavonoids is closely related to their hydroxyl groups. Indeed, the main difference of the structure among neohesperidin, hesperidin and naringin is located at the B-ring, where a methoxy group is in the ortho-position of the hydroxyl group for both neohesperidin and hesperidin. This methoxy group will decrease the activity of hydroxyl group in B-ring due to the steric hindrance and consequently, the binding affinities of both neohesperidin and hesperidin with α-amylase are lower compared to naringin.
All these evidences demonstrated that this flavonoid could prevent the progression of hyperglycaemia in T2DM patients by a complex mechanism that involves the binding of starch, an increase of glycolysis and glycogen concentration, the lower level of gluconeogenesis, an elevating oral glucose tolerance and insulin sensitivity, and decreasing insulin resistance. Moreover, the hydrolysis of starch by amylase is inhibited by vitamin C alone and vitamin C–Cu complex, the latter exerting greater inhibition (Loizzo et al., 2018).

Pancreatic lipase (PL) inhibition is one of the most largely studied mechanisms to combat obesity. The inhibition of this enzyme delays the digestion of triglyceride to absorbable free fatty acids with reduction of postprandial hypertriacylglycerolemia.

All juice samples were able to inhibit the PL enzyme in a concentration-dependent manner as showed in Table 8. A promising hypolipidemic potential was found for JF with IC_{50} value of 179.32 μg/mL followed by JD with IC_{50} value of 192.14 μg/mL. Similar value was observed for JE (IC_{50} value of 197.69 μg/mL).

Pearson's correlation coefficient calculation revealed a positive correlation between chlorogenic acid and lipase enzyme with *r* value of 0.63 (Table 9).

Docking studies of the interaction of flavonoids narirutin, hesperidin, didymin, nobiletin, and tangeretin, and lipase was reported by Zeng et al. (2018). Nobiletin, which had the lowest binding energy, might be the most possible lipase inhibitor.

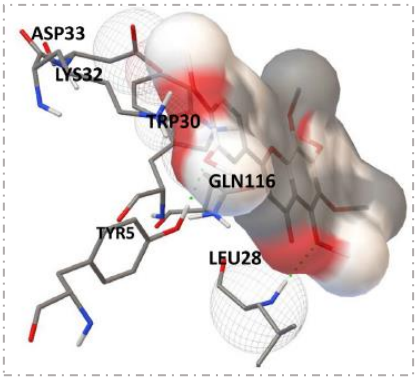


Fig. 8. Molecular docking analysis of nobiletin with lipase. (Adapted from Zeng et al., 2018)

The enzyme has been demonstrated to have an active binding pocket and the amino acid residues of Ser^{152}, Asp^{176} and His^{263} in the pocket that are associated with the catalysis function. But, the molecular docking results indicated that nobiletin did not bind to the key catalytic amino residues despite its low binding energy and strong anti-lipase activity. The authors suggested that nobiletin may bind the protein in a noncovalent interaction to attain its porcine lipase inhibitory activities.

Recently, Casacchia et al. (2019) reported the effect of hybrid Tacle® (TC) a crossbreeding of clementine and Tarocco tetraploids. Results suggested that TC edible portion extract was able to influence anthropometric values, lipid and glucose metabolism on a rat model of obesity and metabolic syndrome and for this reason could be included in dietary supplement for the management of metabolic disorders.

Among *Citrus* flavonoids, Zeng et al. (2018) demonstrated that narirutin and didymin were able to inhibit lipase enzyme with IC_{50} values of 58.98 and 67.30 μg/mL, respectively. Previously, Bustanji et al. (2010) reported the ability of chlorogenic, caffeic and gallic acid, to inhibit the LP and hormone sensitive lipase (HSL) activities in a dose dependent manner, but with different potencies. In fact, gallic acid was found to be the most potent (IC_{50} 10.1 and 14.5 for PL and HSL, respectively) followed by caffeic acid (IC_{50} 32.6 and 40.1 for PL and HSL, respectively). The most promising activity for chlorogenic acid was found against HSL with activity 4.5-times higher in comparison to PL. Recently, Buchholz & Melzig (2015) showed the PL inhibitory activity of hesperidin and neohesperidin and demonstrated that the replacement of rutinose of hesperidin by neohesperidose caused a decrease of the inhibitory activity against PL. Moreover, hydroxy function in position 3′, and methoxy function in position 4′, favoured inhibition.

4.2.6 PCA analysis *Citrus* × *clementina* juices

In the present study, PCA was performed to group and separate the variables analysed in the *C.* × *clementina* juice, obtained from fruits collected in different areas. The loadings of first and second principal components (PC1 and PC2) accounted for 42 and 26% of the variance, respectively (Figure 9).

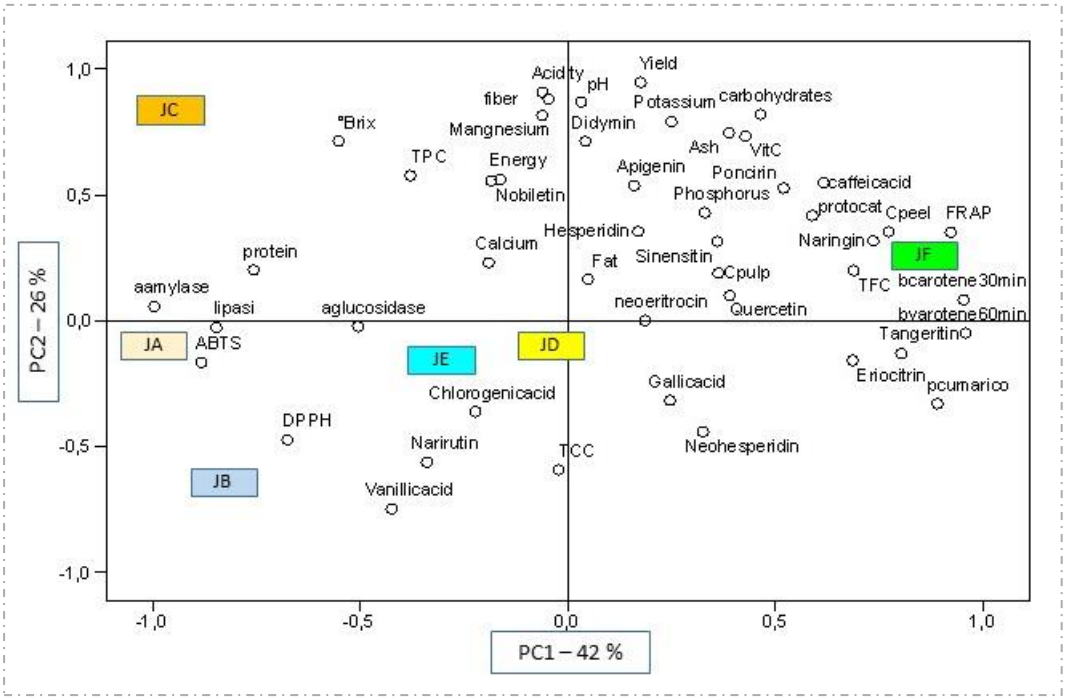


Fig. 9. PCA of juices in function of PC1 and PC2. Score plot and biplot.

The first component (PC1) is highly positively correlated with TFC, eriocitrin, naringin, tangeretin, *p*-coumaric acid, β-carotene-30-min, β-carotene-60-min, FRAP, and juices C*. The PC2 is positively correlated with TPC, vitamin C, didymin, yield, ash, fiber, carbohydrates, pH, acidity, °Brix, potassium and magnesium content.

The scores plot was used to gain an overview of the similarities or differences among the juices. The analysis demonstrated that among the juices analysed JF was located at quadrant top right, which represents the highest TFC, FRAP, β-carotene-bleaching 30 and 60 min, naringin, tangeretin, eriocitrin, sinensetin, and protocatechuic acid content. The JC was located at quadrant top left, which represents the highest TPC, vitamin C, didymin, yield, ash, fiber, carbohydrates, pH, acidity, °Brix, potassium and magnesium. While the other juices (JA, JB, JE and JD) were located at quadrant lower left. Among these JA showed the best features with reference to the variables α-amylase, α-glucosidase and lipase.

PCA confirmed that JF possess the highest bioactive capacity. Thus, the present results provided the basic data for choosing juice with higher antioxidant activity for direct consumption or for production of functional drink.

4.3 *C.* × *clementina* leaves analysis

The study continued on analysis of phytochemical profile, evaluation *in vitro* antioxidant, hypoglycaemic and hypolipidemic activity of *C.* × *clementina* leaves extracts and essential oils in relation to *a)* different geographical area of collection, and *b)* different extraction procedure applied to the matrix. Chemical profiles of extracts and essential oils were investigated by HPLC-DAD and GC-MS, respectively. HPLC analyses were carried

out also in order to exclude the presence of furanocoumarins in the most active *C.* × *clementina* samples.

4.3.1 Extraction yield of *C.* × *clementina* leaves

The extraction yields are reported in Table 10. Generally, samples obtained by Soxhlet extraction procedure showed the highest yield of extraction with 15.00, 13.50, and 12.30% for CO1, RO1 and CE1, respectively followed by maceration with EtOH/H_2O (16.88, 12.44 and 10.23% for CO5, RO5, and CE5, respectively).

It is interesting to note that in disagreement with the literature, the application of the same solvent system *plus* ultrasound assisted maceration process did not determine an increase of extract yields (Medina-Torres et al., 2017). Indeed, ultrasound assisted maceration process with EtOH/H_2O as solvent system produced a yield of 8.27% for CO_2 sample which is higher than that obtained for samples coming from Rosarno and Cetraro with yields of 4.92 and 3.93%, respectively. Similar yield values to Soxhlet extraction were obtained with maceration using EtOH/H_2O as solvent system with percentage 16.88, 12.44 and 10.23% for CO5, RO5 and CE5, respectively.

Yields in the range 0.39-0.43% were obtained for essential oils extracted by hydrodistillation technique.

Table 10. Extraction yield (%) of *C.* × *clementina* samples

Leaves	Sample	Extraction	Yield (%)
Cetraro	CE1	Soxhlet	12.30 ± 9.15^{e}
	CE2	Ultrasound assisted maceration with EtOH/H_2O	3.93 ± 2.90^{l}
	CE3	Ultrasound assisted maceration with EtOH	6.84 ± 4.82^{h}
	CE4	Maceration EtOH	8.78 ± 5.30^{g}
	CE5	Maceration EtOH/H_2O	10.23 ± 8.15^{f}
	CE6	Hydrodistillation	0.39 ± 0.01^{m}
Rosarno	RO1	Soxhlet	13.50 ± 9.35^{b}
	RO2	Ultrasound assisted maceration with EtOH/H_2O	4.92 ± 3.72^{i}
	RO3	Ultrasound assisted maceration with EtOH	4.15 ± 3.44^{li}
	RO4	Maceration EtOH	9.73 ± 7.32^{f}
	RO5	Maceration EtOH/H_2O	12.44 ± 8.15^{de}
	RO6	Hydrodistillation	0.42 ± 0.01^{m}
Corigliano Calabro	CO1	Soxhlet	15.00 ± 10.10^{b}
	CO2	Ultrasound assisted maceration with EtOH/H_2O	8.27 ± 5.35^{g}
	CO3	Ultrasound assisted maceration with EtOH	6.67 ± 4.82^{h}
	CO4	Maceration EtOH	13.22 ± 9.33^{cd}
	CO5	Maceration EtOH/H_2O	16.88 ± 10.60^{a}
	CO6	Hydrodistillation	0.43 ± 0.01^{m}
Sign.			**

Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p < 0.05$. Means in the same column with different small letters differ significantly ($p < 0.05$). Sign: Significant.

4.3.2 *C.* × *clementina* leaves essential oils composition

Constituents of *C.* × *clementina* essential oils were listed in order of their elution time on the HP-5 column in Table 11. A total of thirty-six constituents (96.66% of the total oil) were identified.

Table 11. The main chemical components of *C.* × *clementina* leaves essential oils

Compounds	RI[1]	%			I.M[2]	Sign.
		CE6	RO6	CO6		
α-Thujene	931	1.36 ± 0.11[b]	0.51 ± 0.05[c]	1.43 ± 0.10[a]	1,2,3	**
α-Pinene	938	4.73 ± 0.40[c]	4.93 ± 0.40[b]	5.00 ± 0.41[a]	1,2,3	**
Camphene	953	*tr*	*tr*	*tr*	1,2,3	**
Sabinene	973	22.96 ± 2.21[b]	22.59 ± 2.43[c]	23.32 ± 2.36[a]	1,2,3	**
β-Pinene	980	*tr*	*tr*	*tr*	1,2,3	**
Myrcene	993	4.22 ± 0.43[b]	4.30 ± 0.40[a]	4.45 ± 0.40[a]	1,2,3	**
α-Phellandrene	1005	1.37 ± 0.12[c]	1.59 ± 0.13[a]	1.56 ± 0.21[b]	1,2	**
δ-3-Carene	1009	6.33 ± 0.63[c]	6.62 ± 0.70[b]	7.06 ± 0.75[a]	1,2	**
α-Terpinene	1012	2.41 ± 0.22[b]	2.08 ± 0.22[c]	2.64 ± 0.30[a]	1,2,3	**
Limonene	1030	6.41 ± 0.61[b]	5.88 ± 0.62[c]	6.62 ± 0.63[a]	1,2,3	**
(*E*)-β-Ocimene	1052	6.52 ± 0.60[c]	7.16 ± 0.07[a]	7.02 ± 0.70[b]	1,2	**
γ-Terpinene	1057	3.48 ± 0.36[b]	3.01 ± 0.30[c]	3.72 ± 0.42[a]	1,2,3	**
Terpinolene	1086	2.89 ± 0.22[c]	3.01 ± 0.34[b]	3.14 ± 0.30[a]	1,2,3	**
Linalool	1098	15.80 ± 1.51[b]	16.83 ± 1.66[a]	10.41 ± 1.13[c]	1,2,3	**
Citronellal	1148	2.28 ± 0.23[c]	2.60 ± 0.24[b]	2.87 ± 0.20[a]	1,2	**
Terpinen-4-ol	1176	4.54 ± 0.45[a]	4.07 ± 0.44[b]	2.43 ± 0.26[c]	1,2	**
α-Terpineol	1189	1.40 ± 0.15[a]	1.32 ± 0.11[b]	0.38 ± 0.04[c]	1,2,3	**
Decanal	1205	0.14 ± 0.01[b]	0.17 ± 0.02[a]	0.16 ± 0.02[a]	1,2	**
Nerol	1236	0.81 ± 0.08[b]	1.27 ± 0.15[a]	0.87 ± 0.08[c]	1,2	**
Neral	1242	0.14 ± 0.01[a]	0.16 ± 0.02[a]	*tr*	1,2	**
Geraniol	1255	0.33 ± 0.03[a]	0.11 ± 0.01[b]	*tr*	1,2,3	**
Geranial	1275	0.17 ± 0.01[b]	0.20 ± 0.02[a]	0.12 ± 0.01[c]	1,2,3	**
Neryl acetate	1370	*tr*	*tr*	0.13 ± 0.01[a]	1,2	**
Geranyl acetate	1388	0.23 ± 0.02[b]	0.20 ± 0.02[c]	0.64 ± 0.06[a]	1,2	**
α-Copaene	1377	*tr*	*tr*	*tr*	1,2	**
β-Elemene	1387	0.24 ± 0.02[a]	*tr*	*tr*	1,2	**
β-Cubebene	1387	*tr*	*tr*	*tr*	1,2	**
trans-Caryophyllene	1415	1.04 ± 0.11[b]	0.69 ± 0.06[c]	1.92 ± 0.27[a]	1,2,3	**
(Z)-β-Farnesene	1452	0.62 ± 0.06[b]	0.34 ± 0.03[c]	0.75 ± 0.07[a]	1,2	**
α-Humulene	1454	0.16 ± 0.01[b]	0.11 ± 0.01[c]	0.22 ± 0.02[a]	1,2	**
Germacrene B	1554	0.66 ± 0.07[b]	0.31 ± 0.03[c]	1.04 ± 0.15[a]	1,2	**
δ-cadinene	1526	0.19 ± 0.02[b]	0.10 ± 0.01[c]	0.28 ± 0.02[a]	1,2	**
(*E*)-Nerolidol	1564	0.23 ± 0.02[b]	0.20 ± 0.02[c]	0.35 ± 0.03[a]	1,2	**
β-Sinensal	1697	3.14 ± 0.35[c]	3.30 ± 0.33[b]	4.77 ± 0.42[a]	1,2	**
α-Sinensal	1750	1.46 ± 0.10[c]	1.64 ± 0.24[b]	2.68 ± 0.22[a]	1,2	**
Phytol	1950	0.40 ± 0.03[b]	0.33 ± 0.03[b]	0.88 ± 0.08[a]	1,2	**
Total identified		96.66	95.63	96.86		

Data are reported as mean ± standard deviation (n = 3). [1] Retention indices on the HP 5MS column. [2]IM: identification method: 1 – comparison of retention times; 2 – comparison of mass spectra with MS libraries, 3 – comparison with authentic compounds; *tr*: trace (<0.1%). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same row with different small letters differ significantly (p< 0.05). Sign: significant.

The main volatiles were monoterpene hydrocarbons in which sabinene was the most abundant compound with a percentage of 22.96, 22.59 and 23.32% for the essential oil obtained from CE6, RO6 and CO6, respectively.

Linalool represented the dominant compound of monoterpene hydrocarbons group. This compound showed a similar concentration in CE6 and RO6 with percentage of 15.80 and 16.83%, respectively while a content of 10.40% was observed in Corigliano leaves essential oil. The same trend was found also for terpinen-4-ol, which is in concentration of 4.54 and 4.07% in CE6 e RO6, respectively while a percentage of 2.43 was found for CO6. Moreover, (*E*)-β-ocimene (6.52-7.15%), δ-3-carene (6.32-7.06%) and limonene (5.88-6.62%) are identified in high amount whereas α-pinene and myrcene are present in lower percentages. β-sinensal represented the most abundant in aldehyde components with ranging of 3.14-4.77% respectively, for CE6, RO6 and CO6, respectively.

Results obtained from our analysis have been compared to literature data. Recently, Bonesi et al. (2017) reported the chemical profile of *C.* × *clementina* leaves essential oils, collected in Calabria (Southern Italy). Analysis of data evidenced a similar content of linalool, limonene and δ-3-carene, but a high concentration of sabinene (50.62%) and lower concentration of β-sinensal. A similar content of sabinene and linalool but lower percentages of *δ*-3-carene (0.02%) were found in *C.* × *clementina* leaves from Vietnam (Nguyen et al., 2016).

Previously, Lota et al. (2001) reported the chemical composition of clementine leaves essential oil obtained from different cultivars from Corsica: MA3, Nules, MA2, Hernandina, Tardia Villareal, Reina, Caffin, MacBean, Oroval, Monreal, Bruno, Tomatera, Commune, Marisol, Ragheb and Guillermina. Obtained data confirmed the same order rank: sabinene> linalool> δ-3-carene. In particular, similar values were found for linalool (16.9-23.9%), δ-3-carene for *cultivars* (*cv*) Nules, Reina and Tomatera (6.2-6.5%), citronellal for *cv* Ragheb, Reina and Tomatera (3.0-3.7%) and terpinen-4ol (3.3-4.8%). Sabinene was present in higher percentages (33.1-49.8%) but α-pinene, α-terpinene and myrcene were present in lower percentages in all investigated samples.

El-hawary et al. (2013) reported that limonene (21.13%) was the principal constituents of the essential oil obtained from Egyptian clementine leaves. It was followed by sabinene (16.93%), linalool (12.58%) and δ-3-carene (8.57%).

As evidence, specific climatic and environmental conditions can cause variations in the chemical composition of the vegetable matrix. In fact, the adaptation of many species to the natural environment that hosts them is a fundamental element for the assessment of biodiversity, understood as the chemical, genetic and morphological variability of a plant species (Tundis et al., 2016b). Hence, exogenous and endogenous factors can modify the presence/absence or abundance of a single component within the essential oil (Pitarokili et al., 2003).

4.3.3 Phytochemicals content in *C.* × *clementina* leaves extracts

Table 12 reported the total phenols (TPC), flavonoids (TFC) and carotenoids content (TCC) of *C.* × *clementina* leaves extracts.

Generally, Soxhlet apparatus allowed the extraction of a higher content of these phytochemical classes followed by the maceration in EtOH/H_2O. In particular, RO1 showed a higher TPC with value of 45.54 mg CAE/g FW followed by CO1 (45.36 mg CAE/g FW). Analysis of data obtained by maceration extraction procedure evidenced that a TPC in the range 35.64-42.65 mg CAE/g FW and 23.11-34.98 mg CAE/g FW was observed for EtOH/H_2O and EtOH as solvent system, respectively. A great variability in TPC content was observed by ultrasound assisted maceration procedure.

Both CO1 and RO1 extracts showed high TFC with values of 29.16 and 28.62 mg QE/g FW, respectively. A promising TFC was obtained, also when leaves from Corigliano Calabro were subjected to maceration with EtOH/H_2O (24.98 mg QE/g FW).

Extracts obtained by Soxhlet apparatus showed the highest TCC content in comparison to different extraction techniques with 29.00, 27.30 and 15.66 mg equivalent β-carotene/g FW for RO1, CE1 and CO1, respectively. Significant TCC was recorder also in CE4 and CE5 samples, respectively.

Table 12. Phytochemicals content of *C.* × *clementina* leaves extract

Extracts	Total phenols Content[1]	Total flavonoids Content[2]	Total carotenoids Content[3]
CE1	44.15 ± 2.12^{c}	19.58 ± 0.92^{e}	27.30 ± 1.61^{b}
CE2	13.43 ± 1.40^{q}	5.50 ± 0.53^{p}	8.60 ± 0.81^{n}
CE3	24.25 ± 2.13^{i}	11.79 ± 1.25^{l}	9.89 ± 1.02^{m}
CE4	38.30 ± 3.11^{e}	21.89 ± 1.12^{d}	26.05 ± 2.60^{c}
CE5	23.11 ± 2.02^{l}	10.62 ± 1.01^{m}	25.46 ± 2.5^{d}
RO1	45.54 ± 3.70^{a}	28.62 ± 2.81^{b}	29.00 ± 2.83^{a}
RO2	17.41 ± 1.72^{o}	$6.53 \pm 0.63°$	10.90 ± 1.02^{l}
RO3	14.80 ± 1.50^{p}	$6.83 \pm 0.60°$	11.41 ± 1.10^{gh}
RO4	42.65 ± 4.33^{d}	18.50 ± 1.63^{f}	11.78 ± 1.14^{g}
RO5	34.57 ± 3.21^{n}	16.51 ± 1.81^{h}	10.83 ± 1.05^{i}
CO1	45.36 ± 3.71^{b}	29.16 ± 2.92^{a}	15.66 ± 1.03^{f}
CO2	21.11 ± 2.14^{m}	12.01 ± 1.24^{i}	5.68 ± 0.55^{o}
CO3	18.94 ± 1.84^{n}	7.55 ± 0.71^{n}	5.68 ± 0.52^{o}
CO4	35.64 ± 3.33^{f}	24.98 ± 2.44^{g}	13.27 ± 1.30^{f}
CO5	34.98 ± 3.21^{g}	17.77 ± 1.73^{c}	11.14 ± 1.13^{hi}
Sign.	**	**	**

Data represent means ± SD (standard deviation) (n = 3). [1]mg Chlorogenic Acid Equivalent (CAE)/g fresh weight (FW); [2]mg of Quercetin Equivalents (QE)/g FW. [3]mg β-carotene equivalents/g FW. Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same column with different small letters differ significantly ($p< 0.05$). Sign: significant.

Khettal et al. (2016) investigated the TPC and TFC of aqueous and methanol extracts of *C.* × *clementina* leaves from Algeria and found values of 125.28 mg GAE/g dried weight (DW) and 46.25 mg QE/g DW for aqueous while 11.67 mg GAE/g DW and 7.99 mg QE/g DW where found for methanol extract, respectively.

A perusal analysis of the literature evidenced that no other studies that reported the TPC and TFC in the leaves of *C.* × *clementina* are made so a comparison with different *Citrus* species will be reported. *C. medica cv* Diamante leaves methanol extract obtained by maceration showed a TPC of 401.86 mg GAE/ 100 g FW and a TFC of 97.50 mg QE /100 g FW (Menichini et al., 2011). Values of 137.29 mg CAE/g FW and 58.6 mg QE/g FW were found for TPC and TFC by (Casacchia et al., 2019).

A TPC and TFC of *C. aurantifolia* leaves from Rossano (Calabria, South of Italy) methanol extract of 79.60 mg CAE/g of extract and 43.80 mg QE/g of extract was quantified by Loizzo et al. (2012). To our knowledge, there are no studies concerning the carotenoid content in clementine leaves. Gómez-Mejía et al. (2019) evaluated the TPC and TFC of different *Citrus* species including *C.* × *clementina*. Values of 5.5 mg GAE/g DW and 16.5 mg QE/g DW were found, respectively.

4.3.4 HPLC analysis of *C.* × *clementina* leaves extracts

Phenolic profile

The HPLC-DAD phenolic profile of the extracts obtained from the leaves reported the presence of flavonoids, known as healthy compounds (Loizzo et al., 2018). The results were showed in Table 13, 14, and 15.

Caffeic acid, eriocitrin, rutin, isoquercetin, genistin, hesperidin, poncirin, luteolin, kaempferol, hesperetin, rhamnetin, sinensetin and tangeretin were selected as markers and quantified. Hesperidin is the dominant flavonoid in all investigated extracts with concentration in the range of 174.91-877.00 mg/100 g FW. Noteworthy maceration in $EtOH/H_2O$ was the best technique to hesperidin recovery followed by Soxhlet apparatus. Significant amount of tangeretin (7.24-50.21 mg/100 g FW), sinensetin (4.78-51.32 mg/100 g FW) and eriocitrin (4.36-23.80 mg/100 g FW) were also detected.

A great variability was observed in the content of isoquercetin which is higher in RO1 sample (67.36 mg/100 g FW). A great variability was observed also for rutin that showed the highest content in CO1 extract (114.87 mg/100 g FW). Kaempferol was present only in the extracts obtained by maceration of Cetraro leaves with 1.70 and 0.82 mg/100 g FW for CE4 e CE5, respectively.

Therefore, it emerged that the different chemical composition of the extracts is subject to change under the influence of various factors including climatic and environmental factors, time of collection and applied extraction procedure (M'hiri et al., 2017).

Previously, only Kawaii et al. (2000) reported the phenolic profile of extracts obtained from *C.* × *clementina* leaves collected in Japan and extracted by using methanol: DMSO (1:1). In this case, rutin was the most abundant (885.1 μg/100 mg DW) followed by hesperidin (763.00 μg/100 mg DW). Furthermore, a considerable amount of nobiletin and tangeretin (137.60 and 67.00 μg/100 mg DW, respectively) was found while sinensetin was less concentrated (46.40 μg/100 mg DW).

Table 13. HPLC-DAD of *C.* × *clementina* leaves extracts from Cetraro (mg 100/g FW)

Markers	CE1	CE2	CE3	CE4	CE5	Sign.
Caffeic Acid	0.04 ± 0.01^{d}	0.35 ± 0.04^{bc}	0.29 ± 0.03^{c}	0.80 ± 0.08^{a}	0.50 ± 0.05^{b}	**
Eriocitrin	9.20 ± 0.93^{c}	4.36 ± 0.42^{e}	14.69 ± 1.41^{a}	11.96 ± 1.10^{b}	6.45 ± 0.62^{d}	**
Rutin	68.52 ± 6.30^{a}	4.24 ± 0.43^{e}	60.57 ± 3.42^{b}	6.50 ± 0.65^{d}	7.57 ± 0.83^{c}	**
Isoquercetin	8.95 ± 0.83^{d}	18.71 ± 1.80^{c}	7.73 ± 0.74^{e}	52.01 ± 3.12^{a}	37.35 ± 3.41^{b}	**
Genistin	1.44 ± 0.14^{a}	*nd*	2.3 ± 0.25^{b}	*nd*	*nd*	**
Hesperidin	443.53 ± 14.33^{c}	174.91 ± 10.21^{e}	303.35 ± 11.30^{d}	656.66 ± 15.10^{a}	564.33 ± 12.11^{b}	**
Poncirin	*nd*	*nd*	*nd*	0.46 ± 0.04^{b}	1.17 ± 0.91^{a}	**
Luteolin	*nd*	*nd*	0.94 ± 0.80^{b}	3.14 ± 0.31^{a}	0.69 ± 0.06^{c}	**
Kaempferol	*nd*	*nd*	*nd*	1.70 ± 0.24^{a}	0.82 ± 0.08^{b}	**
Hesperetin	0.31 ± 0.04^{d}	0.10 ± 0.02^{e}	0.52 ± 0.05^{c}	1.42 ± 0.11^{a}	0.66 ± 0.06^{b}	**
Ramnetin	0.43 ± 0.05^{a}	0.19 ± 0.03^{b}	*nd*	0.49 ± 0.05^{a}	0.43 ± 0.04^{a}	**
Sinensetin	26.86 ± 2.62^{b}	7.24 ± 0.76^{e}	16.58 ± 1.62^{d}	35.69 ± 3.05^{a}	23.18 ± 2.3^{c}	***
Tangeretin	26.11 ± 2.50^{c}	7.46 ± 0.70^{e}	22.62 ± 2.24^{d}	41.76 ± 3.51^{a}	40.79 ± 3.51^{b}	**
∑ Indentified Phenolics	585.39	217.56	429.59	812.59	683.94	

Data represent means ± SD (standard deviation) (n = 3). *nd*: not detected. Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p < 0.05$. Means in the same row with different small letters differ significantly ($p < 0.05$). *nd*: not detected. Sign: significant.

Table 14. HPLC-DAD of *C.* × *clementina* leaves extracts from Rosarno (mg 100/g FW)

Markers	RO1	RO2	RO3	RO4	RO5	Sign.
Caffeic Acid	0.86 ± 0.08^{a}	0.03 ± 0.01^{e}	0.40 ± 0.04^{c}	0.67 ± 0.06^{b}	0.32 ± 0.03^{d}	**
Eriocitrin	23.80 ± 2.32^{a}	8.40 ± 0.83^{d}	6.82 ± 0.61^{e}	15.15 ± 1.52^{b}	13.66 ± 1.31^{c}	**
Rutin	16.89 ± 1.61^{c}	4.91 ± 0.43^{e}	34.22 ± 3.22^{b}	73.51 ± 4.91^{a}	9.69 ± 0.92^{d}	**
Isoquercetin	67.36 ± 5.92^{a}	19.41 ± 0.22^{c}	4.22 ± 0.41^{e}	14.34 ± 1.42^{d}	46.81 ± 4.23^{b}	**
Genistin	*nd*	0.41 ± 0.04b	2.14 ± 0.23^{a}	*nd*	*nd*	**
Hesperidin	740.18 ± 15.30^{a}	242.39 ± 10.94^{e}	300.92 ± 11.31^{d}	725.25 ± 15.30^{b}	656.55 ± 15.15^{c}	**
Poncirin	0.65 ± 0.06^{a}	*nd*	*nd*	*nd*	*nd*	**
Luteolin	*nd*	*nd*	0.59 ± 0.05^{a}	*nd*	0.40 ± 0.04^{b}	**
Kaempferol	*nd*	*nd*	*nd*	*nd*	*nd*	**
Hesperetin	0.69 ± 0.06^{b}	0.55 ± 0.05^{c}	*nd*	0.92 ± 0.91^{a}	0.65 ± 0.06^{b}	**
Ramnetin	nd	0.41 ± 0.04^{d}	0.48 ± 0.04^{c}	1.08 ± 0.92^{a}	0.64 ± 0.06^{b}	**
Sinensetin	38.77 ± 3.40^{a}	4.78 ± 0.50^{e}	11.46 ± 1.23^{d}	15.08 ± 1.50^{c}	25.32 ± 2.52^{b}	**
Tangeretin	45.95 ± 4.12^{a}	16.60 ± 1.61^{d}	15.80 ± 1.55^{e}	41.17 ± 3.92^{b}	26.08 ± 2.61^{c}	**
∑ Indentified Phenolics	935.18	297.89	377.05	887.17	780.12	

Data represent means ± SD (standard deviation) (n = 3). *nd*: not detected Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p < 0.05$. Means in the same row with different small letters differ significantly ($p < 0.05$). *nd*: not detected. Sign: significant.

Table 15. HPLC-DAD of *C.* × *clementina* leaves extracts from Corigliano Calabro (mg 100/g FW)

Markers	**CO1**	**CO2**	**CO3**	**CO4**	**CO5**	**Sign.**
Caffeic Acid	0.10 ± 0.02^{d}	*nd*	0.17 ± 0.01^{c}	10.19 ± 1.01^{a}	8.29 ± 2.74^{b}	**
Eriocitrin	16.62 ± 1.61^{a}	5.58 ± 0.51^{e}	6.29 ± 0.63^{d}	9.82 ± 0.92^{b}	6.77 ± 2.52^{c}	**
Rutin	114.87 ± 4.03^{a}	51.14 ± 4.62^{c}	5.56 ± 0.53^{e}	107.63 ± 4.03^{b}	88.11 ± 8.14^{d}	**
Isoquercetin	10.83 ± 1.12^{b}	8.41 ± 0.85^{c}	16.93 ± 1.64^{a}	*nd*	*nd*	**
Genistin	*nd*	*nd*	1.46 ± 0.17^{c}	8.01 ± 0.72^{b}	8.14 ± 2.76^{a}	**
Hesperidin	852.19 ± 16.40^{b}	432.16 ± 14.1^{d}	260.09 ± 12.41^{e}	877.00 ± 16.52^{a}	567.12 ± 15.24^{c}	**
Poncirin	*nd*	0.31 ± 0.03^{a}	*nd*	*nd*	*nd*	**
Luteolin	*nd*	*nd*	*nd*	1.47 ± 0.12^{a}	*nd*	**
Kaempferol	*nd*	*nd*	*nd*	*nd*	*nd*	**
Hesperetin	0.66 ± 0.06^{a}	0.37 ± 0.03^{d}	0.14 ± 0.02^{e}	0.58 ± 0.05^{b}	0.47 ± 0.04^{c}	**
Ramnetin	0.76 ± 0.07^{a}	0.67 ± 0.06^{b}	0.61 ± 0.06^{c}	*nd*	*nd*	**
Sinensetin	30.98 ± 3.03^{c}	17.05 ± 1.74^{d}	0.58 ± 0.05^{e}	46.23 ± 4.30^{b}	51.32 ± 5.10^{a}	**
Tangeretin	32.21 ± 3.21^{c}	16.22 ± 1.64^{d}	12.84 ± 1.23^{e}	50.21 ± 5.03^{b}	43.17 ± 4.32^{a}	**
∑ Indentified Phenolics	1059.22	531.91	304.67	1111.14	773.39	

Data represent means ± SD (standard deviation) (n = 3). *nd*: not detected. Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p < 0.05$. Means in the same row with different small letters differ significantly ($p < 0.05$). *nd*: not detected. Sign: significant.

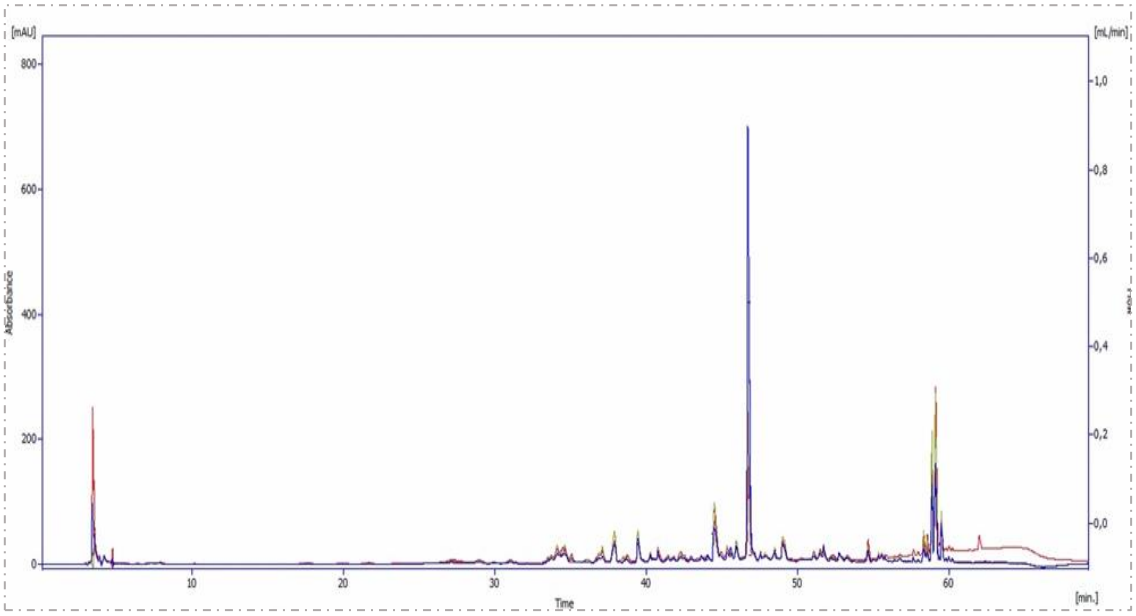


Fig. 10. CO2 phenolic profile

Coumarins determination

The presence of umbelliferone, isopimpinellin and bergapten, was evaluated by HPLC analysis. The applied method showed good specificity, linearity ($r^2 \geq 0.9905$), repeatability (RSDs < 0.02-0.04%) and intermediate precision (RSDs < 0.03-0.07%). For selected markers this method is validated for concentrations ranging from 1.00 to 8.00 mM because recovery values are between tolerance ranges, 97-107% for umbelliferone 84-106% for isopimpinellin and 98-108% for bergapten, which are included in acceptability intervals (75-125%).

Analysis excluded the presence of furanocoumarins in all investigated samples.

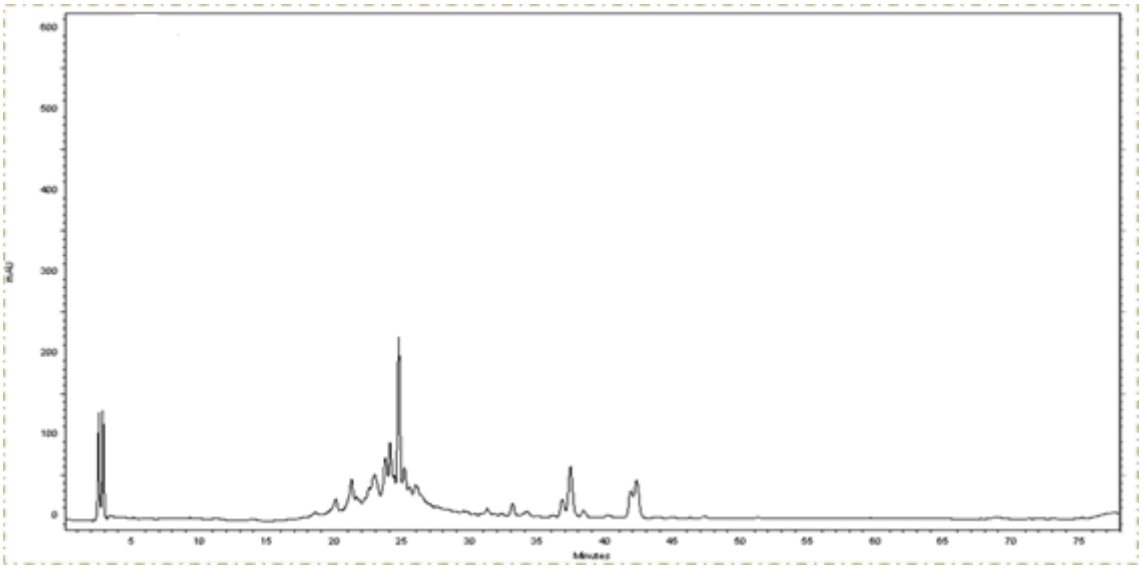


Fig. 11. Coumarins determination in CO2

The results agree with the study conducted by Melough et al. (2017) in which the presence of bergapten in the fruits of clementine was not found. Generally, the coumarins profile is dependent from the species. In particular, bergapten, bergamottin, 5-geranyloxy-7-methoxycoumarin, isopimpinellin, limettin, and oxypeucedanin hydrate were recorded in *C. latifolia* and *C. aurantifolia* extracts. Analysis of the juice of *C. sinensis* cv Changyecheng lead the identification for the first time of isomeranzin and osthole. Meranzin and epoxy-bergamottin were detected in the juice of *C. maxima* (Li et al., 2019).

4.3.5 Antioxidant activity

C. × *clementina* leaves extracts and essential oils antioxidant activity were reported in Table 16 and were expressed in terms of IC_{50}. A concentration-dependent manner was obtained in all tests except FRAP assay. The radical scavenging potential of samples was studied using DPPH and ABTS tests. Those assays are based on an electronic transfer reaction, such as FRAP, DPPH and ABTS tests or on a transfer reaction of a hydrogen atom, such as β-carotene bleaching inhibition test.

Extract obtained by hydroalcoholic maceration of leaves from Corigliano Calabro showed the highest DPPH radical scavenging activity with IC_{50} value of 22.51 μg/mL followed by the extract obtained by the extraction with Soxhlet of the leaves from Cetraro (IC_{50} value of 27.44 μg/mL). Furthermore, interesting results were observed with sample CE3 (IC_{50} value of 29.41 μg/mL).

Generally, in the ABTS assay leaves showed a potent radical scavenging potential. In particular, CO5 showed a better radical scavenging activity than that reported for the positive control ascorbic acid (IC_{50} values of 1.17 and 1.70 μg/mL, respectively). Promising results were obtained, also, with Corigliano Calabro extracts obtained by maceration and ultrasound assisted maceration with EtOH/H_2O with IC_{50} of 1.95 μg/mL for both extracts followed by CO1, RO4 and CE3. The $ABTS^+$ and DPPH are radicals characterized by a different stereochemistry and a different mechanism of generation and after the reaction with the antioxidant compounds, they give a qualitatively different response to the inactivation of their radical.

The potential of *C.* × *clementina* extracts to inhibit lipid peroxidation was assessed using the β-carotene bleaching test. RO3 showed the highest antioxidant activity with IC_{50} values of 3.31 and 6.13 μg/mL respectively after 30 and 60 minutes of incubation. Interesting results were observed for the sample obtained with the same extraction technique but with leaves coming from Corigliano Calabro with IC_{50} values of 3.50 and 6.34 μg/mL after 30 and 60 minutes of incubation, respectively. Interesting, also were the results obtained for CO2 with IC_{50} values of 4.57 and 11.52 μg/mL respectively after 30 and 60 minutes of incubation.

Table 16. *In vitro* antioxidant activity of *C.× clementina* leaves extracts and essential oils

Sample	**DPPH test** IC_{50} (µg/mL)	**ABTS test** IC_{50} (µg/mL)	**β-carotene bleaching test t 30 min** IC_{50} (µg/ml)	**β-carotene bleaching test t 60 min** IC_{50} (µg/mL)	**FRAP test** µM Fe (II)/g
CE1	$27.44 \pm 1.20^{****}$	$3.91 \pm 0.34^{**}$	$34.49 \pm 2.23^{****}$	$26.61 \pm 2.22^{****}$	$53.15 \pm 2.05^{****}$
CE2	$57.42 \pm 3.12^{****}$	$9.98 \pm 1.23^{****}$	$86.22 \pm 3.41^{****}$	$40.22 \pm 2.51^{****}$	64.25 ± 2.62^{ns}
CE3	$29.41 \pm 1.34^{****}$	2.35 ± 0.22^{ns}	$37.21 \pm 2.42^{****}$	$24.45 \pm 2.16^{****}$	60.94 ± 2.42^{ns}
CE4	$36.90 \pm 1.90^{****}$	2.75 ± 0.21^{ns}	$85.36 \pm 3.23^{****}$	$55.23 \pm 3.11^{****}$	$52.83 \pm 2.13^{****}$
CE5	$51.61 \pm 2.20^{****}$	$9.09 \pm 0.85^{****}$	$6.91 \pm 0.63^{**}$	$6.43 \pm 0.64^{*}$	$52.94 \pm 2.21^{****}$
CE6	$301.8 \pm 5.33^{****}$	$9.44 \pm 0.87^{****}$	$50.91 \pm 2.77^{****}$	$85.07 \pm 4.47^{****}$	$32.01 \pm 1.81^{****}$
RO1	$63.25 \pm 2.61^{****}$	3.15 ± 0.20^{ns}	$75.78 \pm 2.80^{****}$	$76.63 \pm 4.14^{****}$	$54.95 \pm 1.91^{**}$
RO2	$59.37 \pm 2.51^{****}$	$4.71 \pm 0.31^{***}$	$21.36 \pm 2.14^{****}$	$16.94 \pm 1.54^{****}$	$53.78 \pm 2.41^{***}$
RO3	$55.49 \pm 2.33^{****}$	$5.92 \pm 0.52^{****}$	3.31 ± 0.30^{ns}	$6.13 \pm 0.74^{*}$	61.24 ± 2.72^{ns}
RO4	$83.14 \pm 3.41^{****}$	2.35 ± 0.23^{ns}	$7.28 \pm 0.71^{**}$	$8.12 \pm 0.82^{**}$	$47.64 \pm 2.02^{****}$
RO5	$46.76 \pm 2.10^{****}$	$5.52 \pm 0.51^{****}$	$5.64 \pm 0.52^{**}$	$9.25 \pm 0.90^{***}$	64.21 ± 2.42^{ns}
RO6	$478.21 \pm 6.10^{****}$	$9.79 \pm 0.82^{****}$	4.76 ± 0.44^{ns}	$15.07 \pm 1.23^{****}$	$20.69 \pm 0.85^{****}$
CO1	$40.25 \pm 1.62^{****}$	2.35 ± 0.20^{ns}	$5.93 \pm 0.51^{*}$	$29.05 \pm 0.24^{****}$	$54.29 \pm 2.04^{***}$
CO2	$32.37 \pm 1.40^{****}$	1.95 ± 0.11^{ns}	4.57 ± 0.42^{ns}	$11.52 \pm 0.95^{****}$	$45.77 \pm 1.41^{****}$
CO3	$57.43 \pm 3.13^{****}$	3.15 ± 0.33^{ns}	3.53 ± 0.32^{ns}	$6.34 \pm 0.60^{*}$	$46.57 \pm 1.81^{****}$
CO4	$22.51 \pm 1.80^{****}$	1.95 ± 0.11^{ns}	$7.18 \pm 0.75^{**}$	$22.01 \pm 2.11^{****}$	61.42 ± 2.24^{ns}
CO5	$39.97 \pm 2.31^{****}$	1.17 ± 0.12^{ns}	$86.29 \pm 3.14^{****}$	$91.17 \pm 4.02^{****}$	$51.74 \pm 2.42^{****}$
CO6	$556.12 \pm 7.30^{****}$	$27.25 \pm 2.22^{****}$	$92.96 \pm 4.12^{****}$	$94.18 \pm 4.21^{****}$	$32.26 \pm 1.22^{****}$
Positive control					
Ascorbic acid	5.01 ± 0.80	1.72 ± 0.06			
Propyl gallate			0.09 ± 0.004	0.09 ± 0.004	
BHT					63.23 ± 4.31

Data are expressed as means ± S.D. (n= 3). DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$); β-carotene bleaching test; Ferric Reducing Antioxidant Power (FRAP); Relative Antioxidant Capacity Index (RACI) and Global Antioxidant Score (GAS). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p< 0.01, *p< 0.1 compared with the positive controls. ns: not significant

Samples were tested also in the FRAP test. In this assay the extract obtained by maceration and ultrasound assisted maceration with EtOH of Corigliano Calabro leaves showed the highest antioxidant activity with values both of 64.21 μM Fe (II)/g followed by extract obtained by Cetraro leaves extracted with hydro-alcoholic ultrasound assisted maceration (64.25 μM Fe (II)/g). These extracts presented values highest compared to positive control. An interesting ferric reducing power was found, also, with CE3, RO3 and CO4 with values of 60.94, 61.24 and 61.42 μM Fe (II)/g, respectively. These FRAP values are comparable to that found for the BHT positive control.

Pearson's correlation coefficient was calculated and positive values are reported in Table 17. Sinensetin content exhibited a positive *Pearson*'s correlation coefficient with β-carotene bleaching test (after 60 min of incubation) with *r* value of 0.67.

Table 17. *Pearson*'s correlation between phenolic compounds content in investigated samples and activity

	DPPH test	ABTS test	β-carotene bleaching test t 30 min	β-carotene bleaching test t 60 min	FRAP test
TPC			0.15	0.40	
TFC			0.15	0.45	
TCC	0.04	0.06	0.30		
Caffeic acid			0.17	0.40	0.14
Eriocitrin	0.17		0.11	0.30	0.07
Rutin				0.12	
Isoquercetin	0.31	0.24	0.32	0.25	0.05
Genistein			0.13	0.35	0.15
Hesperidin			0.23		
Poncirin	0.09	0.33	0.10	0.09	
Luteolin			0.23	0.10	0.19
Kaempherol		0.12	0.32	0.15	
Hesperitin			0.22	0.25	
Ramnetin	0.42				
Sinensetin			0.37	0.67	0.08
Tangeretin			0.18	0.44	

Concerning essential oils activity, several differences were displayed. In DPPH assay, CE6 presented a radical scavenging activity 1.8-times higher in comparison to CO6 whereas similar values were recorded in ABTS. Promising results, on protection of lipid peroxidation, was observed with RO6 essential oil that showed IC_{50} values of 4.76 and 17.07 μg/mL after 30 and 60 minutes of incubation, respectively. These data disagreed with those reported by Bonesi et al. (2017) in which higher IC_{50} values were found. The difference of antioxidant activity can be related to a different synergy between the various constituents or that the activity of the main constituents was modulated by less abundant compounds.

Pearson's correlation coefficient was calculated between EO constituents and antioxidant activity (Table 18). The most significant positive correlations were found between α-pinene, citronellal and terpinolene content and DPPH test with *r* = 1.00, 0.99 and 0.97, respectively. Also, δ-3-carene, myrcene and α-phellandrene showed interesting

positive correlation (0.94, 0.92 and 0.91, respectively). A r = 1.00 was found for neryl acetate, geranyl acetate, and β-sinensal and ABTS. Similar value correlations were found for α-synensal, phytol and nerolidol. In addition, sabinene, α-terpinene, α-humulene, germacrene B and δ-cadinene exhibited a positive correlation with β-carotene bleaching test after 30 min of incubation with r value of 1.00. Positive correlations with β-carotene bleaching test, after 60 min of incubation, and α-pinene (r = 1.00), limonene and Z-β-farnesene (r = 0.98) were found. Additionally, the same compounds together with sabinene showed positive correlations with FRAP test (1.00, 1.00, 0.97 and 0.96, respectively for sabinene, α-pinene, limonene and Z-β-farnesene).

Table 18. *Pearson*'s correlation between EO constituents and activity

	DPPH test	ABTS test	β-carotene bleaching test t 30 min	β-carotene bleaching test t 60 min	FRAP test
α-Thujene		0.54	0.91	1.00	1.00
α-Pinene	1.00	0.71	0.22		
Sabinene	0.29	0.85	1.00	0.92	0.88
Myrcene	0.92	0.95	0.62	0.28	0.19
α-Phellandrene	0.91	0.40			
δ-3-Carene	0.94	0.93	0.58	0.22	0.14
α-Terpinene	0.20	0.80	1.00	0.95	0.92
Limonene	0.06	0.71	0.98	0.98	0.97
(*E*)-β-Ocimene	0.87	0.33			
γ-Terpinene	0.12	0.75	0.99	0.97	0.95
Terpinolene	0.97	0.89	0.50	0.13	0.04
Citronellal	0.99	0.85	0.43	0.06	
Decanal	0.80	0.21	0.85	0.59	0.89
Nerol	0.33				
Geraniol			0.08	0.17	
Neryl acetate	0.74	1.00	0.85	0.59	0.52
Geranyl acetate	0.69	1.00	0.88	0.64	0.57
β-Elemene			0.03	0.41	0.48
trans-Caryophyllene	0.52	0.96	0.96	0.79	0.73
(Z)-β-Farnesene	0.10	0.73	0.98	0.98	0.96
α-Humulene	0.35	0.88	1.00	0.89	0.85
Germacrene B	0.32	0.87	1.00	0.90	0.86
δ-cadinene	0.30	0.86	1.00	0.91	0.88
(*E*)-Nerolidol	0.59	0.98	0.94	0.73	0.67
β-Sinensal	0.79	1.00	0.80	0.51	0.44
α-Sinensal	0.82	0.99	0.77	0.47	0.39
Phytol	0.65	0.99	0.91	0.68	0.61

Previously, Khettal et al. (2016) demonstrated a ferric reducing power of 30.60 and 13.85 mg BHAE/g of dry matter for the aqueous and methanol extracts of *C.* × *clementina* leaves from Algeria. In addition, the same samples showed in the DPPH test, IC_{50} values of 43.40 and 41.85 μg/mL respectively, whereas values of 1174.43 and 378.63 μM equivalents Trolox/g of dry matter were reported for ABTS.

A promising antioxidant potential was found also for *C. aurantifolia* leaves methanol and *n*-hexane extracts (Loizzo et al., 2012). No significant differences were observed between the two applied solvent systems in terms of protection of lipid peroxidation whereas in FRAP assay the *n*-hexane extract showed the highest ferric reducing power (205.40 μM Fe (II)/g). Cilla et al. (2018) demonstrated the protective effect of pulp bio-accessible fractions of oranges from Navel and Cara oranges cultivars as well as Clementine mandarin freshly harvested and refrigerated stored against oxidative stress. These fractions act by pre-serving cell viability, correct cell cycle progression, mitochondrial membrane potential and diminishing Reactive Oxygen Species (ROS) level and lipid peroxidation. More recently, *C. lumia* albedo was investigated for its antioxidant potential by using different *in vitro* methods and showed the following trend: Oxygen radical Absorbance capacity (ORAC) > FRAP > Folin-Ciocalteu > Trolox Equivalent antioxidant capacity (TEAC) > β-carotene bleaching > DPPH (Smeriglio et al. 2019).

The RACI of each sample is a scientific combination of data from different antioxidant methods without unit limitations and no variance between the methods and makes comparison of the antioxidant capacity of the matrix probable. Data obtained from the DPPH, ABTS, FRAP and β-carotene bleaching tests were used to calculate the RACI values for each sample. Figure 12 reported RACI values of all investigated samples. ultrasound assisted maceration with EtOH/H_2O of leaves from Corigliano Calabro (CO2) exhibited the greatest antioxidant potential followed by CE2 obtained with the same process but by the extraction of plant collected in Cetraro. Promising results were obtained also with CO3 that was obtained using the same technology but with EtOH as solvent that showed a RACI value of -0.58.

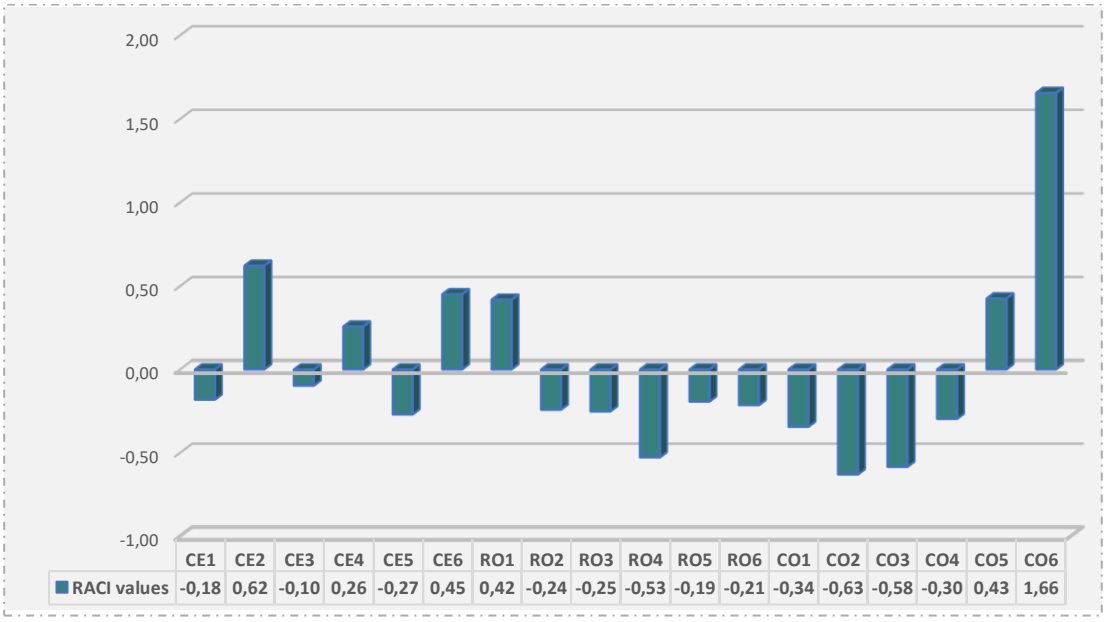


Fig. 12. RACI values of *C.* × *clementina* leaves extracts and essential oils.

4.3.6 Leaves extracts and essential oils enzymes inhibitory activity

Following our previously investigation regarding the hypoglycaemic potential of *Citrus* derived products and by-products herein we reported the study i*n vitro* hypoglycaemic potential of *C.* × *clementina* extracts and essential oil. For this purpose, the study on the inhibition of the enzymes responsible for the cleavage of carbohydrates such as α-amylase and α-glucosidase are applied. Results are reported in Table 19.

An inhibition in a concentration-dependent manner was observed for all investigated samples. Samples CE2 showed the highest α-amylase inhibitory activity with IC_{50} value of 64.37 μg/mL followed by CO2 and CE3 (IC_{50} values of 73.90 and 74.69 μg/mL, respectively). In *α*-glucosidase test, the greatest hypoglycaemic activity was obtained through ultrasound assisted maceration using EtOH as solvent system with IC_{50} values of 51.61, 55.02 and 58.40 μg/mL, for RO3, CO3 and CE3, respectively.

Table 19. Hypoglicaemic and hypolipidemic activity of *C.*× *clementina* leaves extracts and essential oils

Sample	**α-Amylase** IC_{50} **(μg/mL)**	**α-Glucosidase** IC_{50} **(μg/mL)**	**Lipase** IC_{50} **(μg/mL)**
CE1	$81.26 \pm 3.62^{****}$	$60.33 \pm 2.72^{*}$	$137.45 \pm 4.33^{****}$
CE2	$64.37 \pm 2.53^{****}$	$131.13 \pm 4.33^{****}$	$114.37 \pm 4.24^{****}$
CE3	$74.69 \pm 2.72^{****}$	58.40 ± 3.63^{ns}	$109.97 \pm 4.17^{****}$
CE4	$98.16 \pm 3.94^{****}$	$156.93 \pm 4.42^{****}$	$198.74 \pm 4.52^{****}$
CE5	$247.61 \pm 5.05^{****}$	$119.59 \pm 4.12^{****}$	$201.32 \pm 4.54^{****}$
CE6	$141.64 \pm 4.32^{****}$	$282.65 \pm 5.25^{****}$	$175.56 \pm 4.31^{****}$
RO1	$138.13 \pm 4.25^{****}$	$70.94 \pm 2.84^{****}$	$145.88 \pm 4.36^{****}$
RO2	$85.96 \pm 3.78^{****}$	$116.96 \pm 4.12^{****}$	$124.58 \pm 4.26^{****}$
RO3	$77.51 \pm 2.61^{****}$	51.61 ± 2.46^{ns}	$110.37 \pm 4.13^{****}$
RO4	$70.00 \pm 2.59^{****}$	$91.59 \pm 3.92^{****}$	$189.73 \pm 4.31^{****}$
RO5	$177.55 \pm 4.74^{****}$	$166.89 \pm 4.55^{****}$	$191.19 \pm 4.53^{****}$
RO6	$135.51 \pm 4.23^{****}$	$151.27 \pm 4.25^{****}$	$183.27 \pm 4.33^{****}$
CO1	$86.89 \pm 3.63^{****}$	$75.29 \pm 2.81^{****}$	$125.74 \pm 4.51^{****}$
CO2	$73.91 \pm 2.75^{****}$	$103.79 \pm 4.09^{****}$	$104.45 \pm 4.11^{****}$
CO3	$68.13 \pm 2.82^{****}$	55.02 ± 3.69^{ns}	$109.73 \pm 4.13^{****}$
CO4	$101.91 \pm 4.02^{****}$	$215.32 \pm 4.91^{****}$	$179.94 \pm 4.24^{****}$
CO5	$135.41 \pm 4.15^{****}$	$85.79 \pm 2.52^{****}$	$195.47 \pm 4.53^{****}$
CO6	$148.64 \pm 4.43^{****}$	$225.72 \pm 4.93^{****}$	$181.33 \pm 4.28^{****}$
Positive control			
Acarbose	50.01 ± 0.92	35.52 ± 1.23	
Orlistat			37.53 ± 0.92

Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the positive control. ns: not significant.

Pearson's correlation coefficient analysis revealed a positive correlation between poncirin, isoquercetin, and *α*-amylase with *r* value of 0.72, and 0.50, respectively. Additionally, luteolin showed a positive correlation with *α*-glucosidase (r value of 0.54).

Recently, Martinez-Gonzalez (2019) reported the analysis of possible binding sites for flavonoids on pancreatic α-amylase. Hydrogen binding, hydrophobic binding and Van der Waals forces were the main interactions between α-amylase and these compounds. Flavonoids may bind Trp^{58}, Trp^{59}, Tyr^{62}, Gln^{63} and Asp^{197} in the active site of the enzyme. The enzyme-luteolin complex was stabilized through two bindings types, hydrogen and hydrophobic, with Trp59, and one hydrogen binding interaction with Gln^{63}.

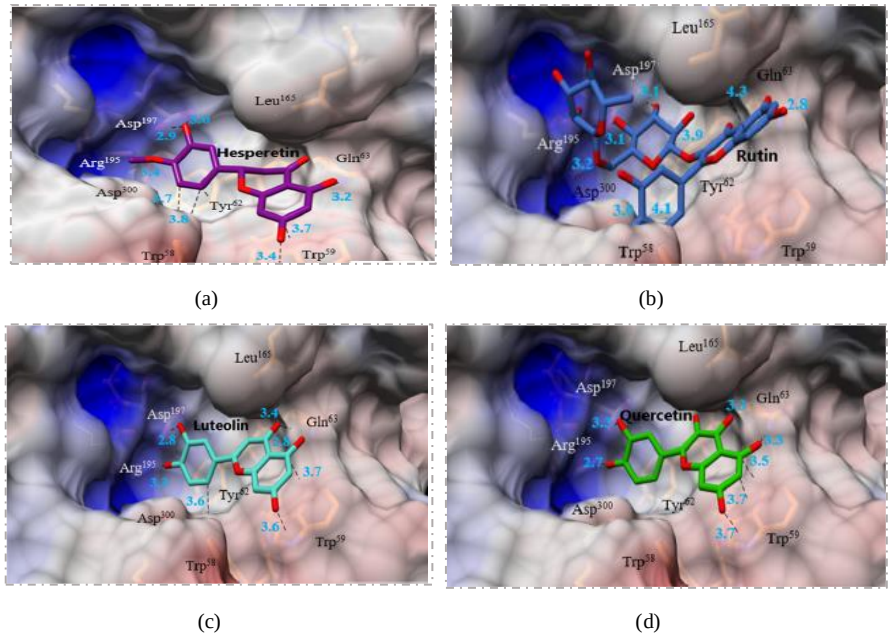


Fig. 13. Molecular docking of hesperitin (a), rutin (b), luteolin (c), and quercetin with α-amylase. (Adapted from Martinez-Gonzalez, 2019)

Quercetin showed hydrogen binding with Gln^{63}, and Van der Waals forces with Leu^{165}. The enzyme-rutin complex included different amino acids such as Val^{163}, Glu^{233} and Asp^{300}. Asp^{300}-rutin interaction maybe related to a higher affinity for the flavonoid. Hesperitin showed hydrophobic binding with Trp^{58}, Trp^{59}, Tyr^{62} and hydrogen binding with Gln^{63} and Asp^{197}. Casacchia et al. (2019) found that both naringenin and hesperetin interact with the three catalytic residues in the enzyme active site. In particular, Asp^{197} is the key residue for the nucleophilic hydrolytic catalysis of α-amylase on starch polymers. The interaction with Glu^{233} precludes its catalytic function as acid-base promoter of hydrolysis, while the binding with Asp^{300} prevents the correct orientation of starch molecule into the active site. The binding energies calculated for naringenin and hesperetin were -8.1 and -7.7 kcal/mol, respectively, indicating a good affinity for the protein. He et al. (2019) found that the active site of α-glucosidase was located inside the enzyme molecule. As a result, when the flavonoid glycosides with a larger molecular volume entered, the steric hindrance to be overcome was greater than that of flavonoid aglycones, so the inhibitory performance of flavonoid glycosides was generally lower. Additionally, they reported that amino acid residues Asp^{215} was involved in the hydrogen bonding interaction between each flavonoid (quercetin, luteolin, hesperitin, naringenin,

hesperidin and rutin) with the enzyme and the number of phenolic hydroxyl groups on the B ring was positively correlated with inhibitory activity. Previously, Tadera et el. (2006) reported a comparison of inhibitor activity of 4-hydroxylates, 4,5-hydroxylates and 3,4,5-hydroxylates flavonoids. The inhibition increased with an increase of hydroxyl groups on the B ring (Isoflavone > flavanol >flavone > flavanone > flavanone-3-olo).

A promising hypolipidemic potential against lipase was found for CO2 with IC_{50} value of 104.45 µg/mL followed by CO3 with IC_{50} value of 109.73 µg/mL. Similar value was observed for CE3.

Pearson's correlation coefficient revealed a positive correlation between tangeretin, hesperitin, and hesperidin content and lipase enzyme with *r* value of 0.79, 0.67, and 0.63 respectively (Table 20).

Table 20. *Pearson*'s correlation between phenolic compounds content in investigated sample and activity

	α-Amylase	**α-Glucosidase**	**Lipase**
TPC	0.13		0.49
TFC	0.14	0.10	0.40
TCC	0.40		0.47
Caffeic acid	0.12	0.34	0.44
Eriocitrin	0.08		0.06
Rutin			0.06
Isoquercetin	0.50	0.20	0.35
Genistein		0.12	0.24
Hesperidin	0.34	0.31	0.63
Poncirin	0.72	0.07	0.36
Luteolin	0.09	0.54	0.42
Kaempherol	0.31	0.32	0.50
Hesperitin	0.26	0.37	0.67
Ramnetin			0.03
Sinensetin	0.37	0.17	0.61
Tangeretin	0.47	0.23	0.79

Casacchia et al. (2019) described the crystallographic structure of lipase that includes three structural domains: N-terminal domain containing the active site, non-catalytic C-terminus encompassing a colipase binding site, and a lid loop that regulates the ligand entry into the active site. A catalytic triad (Ser^{152}, Asp^{176} and Hys^{263}) actively participates to lipid hydrolysis in the active site. Both naringenin and hesperetin were found to interact with all these residues.

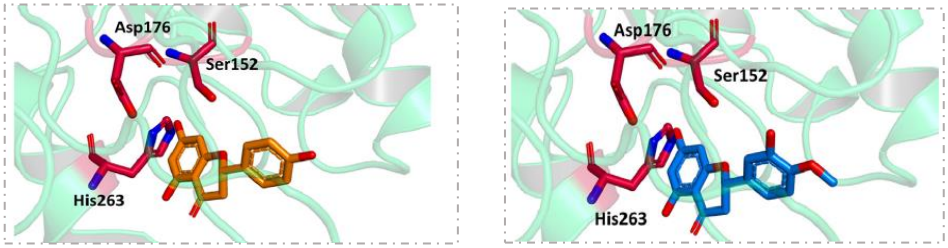


Fig. 14. Naringenin (yellow) and hesperetin (blue) anchored within the binding site of lipase. (Adapted from Casacchia et al, 2019)

Regarding essential oil, all samples showed could inhibit both enzymes in a concentration-dependent manner, but the most promising activity was found against α-amylase enzyme. Additionally, in α-amylase assay, CE6 and RO6 demonstrated greater activity in comparison to extracts obtained by maceration (EtOH). The same observation could be done for lipase inhibition assay, and in particular, RO6 was 1.6-times more active than RO5.

Pearson's correlation coefficient data revealed a positive correlation between sabinene α-humulene, germacrene B and δ-cadinene content and *α*-amylase with *r* value of 1.00, respectively (Table 21). The interesting positive correlations were also found between α-terpinene, limonene, γ-terpinene, and *trans*-caryophyllene. The analysis revealed that, α-thujene, β-elemene, and limonene were positively correlated with *α*-glucosidase. Moreover, (*E*)-β-ocimene, decanal and α-phellandrene presented positive correlations with lipase (*r* = 1.00, 1.00 and 0.99, respectively). Interesting, also, the correlation between α-pinene and lipase with *r* value of 0.88.

Table 21. Positive *Pearson*'s correlation between EO constituents and activity

	α-Amylase	**α-Glucosidase**	**Lipase**
α-Thujene	0.88	0.87	
α-Pinene	0.29		0.88
Sabinene	1.00	0.57	
Myrcene	0.68		0.59
α-Phellandrene			0.99
δ-3-Carene	0.63		0.63
α-Terpinene	0.99	0.65	
Limonene	0.96	0.75	
(*E*)-β-Ocimene			1.00
γ-Terpinene	0.97	0.71	
Terpinolene	0.56		0.70
Citronellal	0.50		0.75
Decanal			1.00
Nerol			0.78
Geraniol		0.59	
Neryl acetate	0.89	0.08	0.28
Geranyl acetate	0.91	0.14	0.22
β-Elemene		0.83	
trans-Caryophyllene	0.98	0.35	
(Z)-β-Farnesene	0.97	0.72	
α-Humulene	1.00	0.52	
Germacrene B	1.00	0.55	
δ-cadinene	1.00	0.57	
(*E*)-Nerolidol	0.96	0.26	0.09
β-Sinensal	0.84		0.36
α-Sinensal	0.82		0.40
Phytol	0.94	0.19	0.16

In recent decades, several *in vitro* and *in vivo* studies demonstrated the importance of genus *Citrus* in the prevention of T2DM and obesity.

Menichini et al. (2011) reported the α-amylase and α-glucosidase inhibitory activities of hydroalcoholic maceration of *C. medica* cv. Diamante leaves and found IC_{50} values of 438.50 and 777.80 μg/mL, respectively. A moderate inhibitory activity against α-amylase was observed for fruit of *C. macroptera* with IC_{50} value of 3.63 mg/mL (Uddin et al., 2014).

Oboh & Ademosun (2011a) reported the extraction of free phenols from *C. maxima* peels by 80% acetone, while the bound phenols were extracted by the alkaline and acidic hydrolysis of the residue with ethyl acetate. These extracts were tested against carbohydrate hydrolysing enzymes. Comparison of data evidenced that phenols from peels are able to exert a more pronounced activity against α-glucosidase in agreement with those reported for *C. paradisi* and *C. sinensis* peels extract (Oboh & Ademosun 2011b, c). This prevalent action on α-glucosidase enzyme agrees with our data and has a certain importance since glucosidase inhibitors present lower side effect associated to the use of drugs such as acarbose or voglibose. *C. sinensis* peels demonstrated to optimize glucose uptake in high-fat diet-fed streptozotocin-induced insulin-resistant diabetic rats through the insulin-dependent signalling cascade mechanism (Sathiyabama et al., 2018).

Recently, the anti-lipase activity of *C. reticulata* extracts collected at different times was evaluated (Zeng et al., 2018). These extracts were able to inhibit the lipase enzyme in a concentration-dependent manner and showed IC_{50} values in the range between 0.383 and 1.370 mg/mL. Basli et al. (2016) reported that lemon extract inhibited lipase with IC_{50} value of 110.07 μg/mL. Moreover, *C. unshiu* peels extract showed inhibitory effect on lipase activity with IC_{50} value of 507.01 μg/mL (Kim et al., 2016). Previously, IC_{50} values in the range of 0.25-0.36 mg/mL were found for the pulp extract of the six pomelo cultivars against pancreatic lipase (Makynem et al., 2013).

Among identified constituents, luteolin showed α-amylase inhibitory activity with IC_{50} value of 18.00 μM. The hypoglycaemic effect was proved for poncirin that showed IC_{50} values of 125.5 and 8.0 μM against α-amylase and α-glucosidase, respectively (Tundis et al., 2016a). Values of 12.70, 1.00, 6.00 and 14.80 μM were found for quercetin, poncirin, hesperidin and tangeretin (Sahnoun et al., 2017; Martinez-Gonzalez et al., 2018). The same flavonoids showed a lower potency in α-glucosidase inhibitory activity with IC_{50} values of 125.50, 111.00 and 141.00 μM for poncirin, hesperidin and tangeretin, respectively (Sahnoun et al., 2017).

Furthermore, in streptozotocin induced diabetic rats, hesperidin ameliorates hyperglycaemia by regulating key enzymes of carbohydrates metabolism such as fructose-1,6-bisphosphatase, hexokinase, glucose-6-phosphate dehydrogenase, and glucose-6-phosphatase (Sundaram et al., 2019). Additionally, hesperidin showed an IC_{50} value of 52.4 μM against lipase enzyme.

Ghorbani (2017) described anti-diabetic property of rutin. In STZ-induced diabetic rats, oral administration of 50 or 100 mg/kg of this compound decreased fasting blood glucose as well as HbA1c levels. Moreover, chronic administration of 200 mg/kg of rutin, determined a reduction (30–40%) of prevalence of diabetes in STZ-treated mice. In

addition, more recently Gupta et al. (2019) demonstrated that rutin treatment (50 mg/kg) for 24 weeks arrested the biochemical disturbances of diabetic retinopathy lowering VEGF, TNF-α, and increasing total antioxidant capacity in the retina. The positive effect of rutin on lipid profile was reported by Wang et al. (2015). This compound decreased serum levels of triglycerides, very low-density lipoproteins (VLDL), low density lipoproteins (LDL) and increased the level of high-density lipoproteins (HDL) in different models of DM.

Mohamed et al. (2012) reported the ability of sinensetin compound to show inhibitory activity on α-glucosidase (IC_{50}: 0.66 mg/ml) and α-amylase (IC_{50}: 1.13 mg/ml). Also, sinensetin increased the phosphorylation of protein kinase A and hormone-sensitive lipase, indicating its lipolytic property via a cAMP-mediated signalling pathway. This compound also upregulated mRNA expression of carnitine palmitoyltransferase-1α, suggesting that sinensetin enhances fatty acid β-oxidation through the AMPK pathway (Kang et al., 2013).

4.3.7 PCA leaves extracts and essential oil

The first two principal components (PCs) explained 70 % of the total variance observed in the original variables. In particular, PC1 explains 48 %, while PC2 explains 22 %.

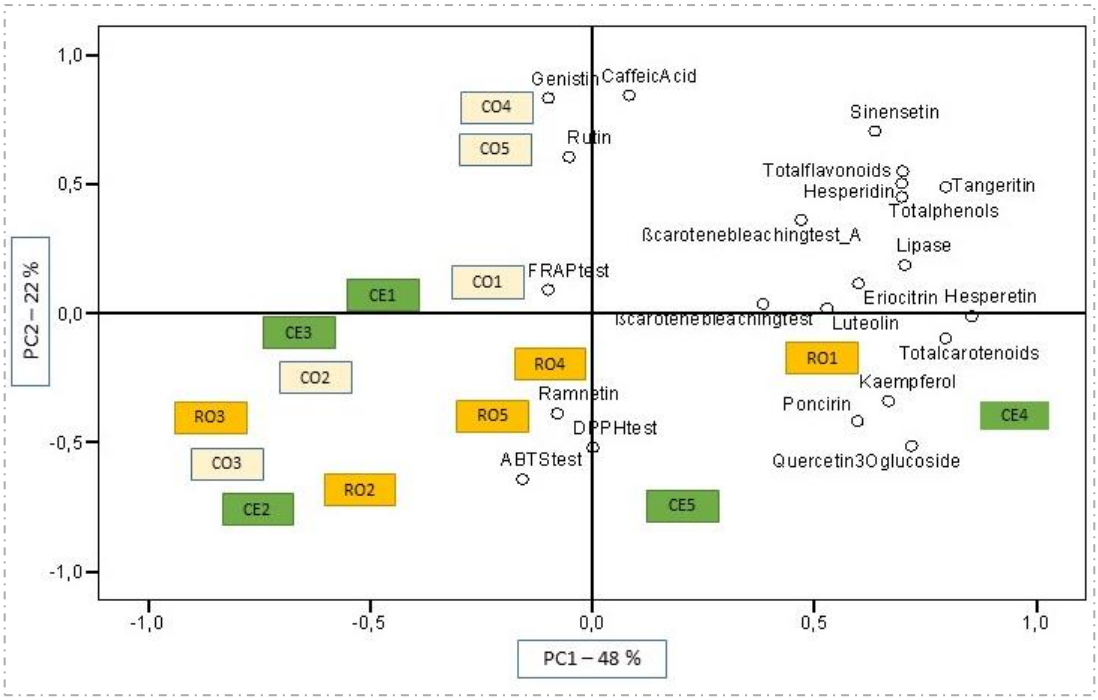


Fig. 15. Scatter plot (PC1 and PC2) on the main sources of variability between the samples of leaves extracted using different methods.

Figure 15 showed two main principal components (PCs) characterized the TPC, TFC, TCC, eriocitrin, isoquercetin, ponirin, luteolin, kaempferol, hesperidin, sinensetin, tangeretin, lipase, and β-carotene bleaching test after 30 min and 60 min incubation,

caffeic acid, rutin, genistin and sinensetin of the extracts (obtained from Soxhlet apparatus, ultrasound assisted maceration with EtOH, ultrasound assisted maceration with EtOH/H_2O, maceration with EtOH/H_2O and maceration with EtOH alone), obtained from leaves collected in different area of Calabria.

Figure 15 also showed that PC1, was positively correlated with the following variables: TPC, TFC, TCC, eriocitrin, isoquercitrin, hesperidin, poncirin, luteolin, kaempferol, hesperidin, sinensetin, tangeretin, lipase, β-carotene bleaching test after 30 min and 60 min incubation while PC2 was positively correlated with caffeic acid, rutin, genistein and sinensetin.

Moreover, PCA analysis was performed in order to classify the extraction methods used: Soxhlet, ultrasound maceration with EtOH, ultrasound maceration with EtOH/H_2O, maceration with EtOH/H_2O and maceration with EtOH, based on 22 variables.

As showed in Figure 15 extracts obtained by Soxhlet apparatus, maceration with EtOH and EtOH/H_2O were separated from the extracts obtained by ultrasound assisted maceration with both EtOH and EtOH/H_2O. This was due to the higher concentration of certain phytochemicals such as hesperidin, caffeic acid, sinensetin, rutin, tangeretin and luteolin.

4.4 *C.* × *clementina* peels analysis

A perusal analysis of the literature revealed that clementine peels are less investigated. In this context, in the present project we have investigated a model for the reuse of *C.* × *clementina* peels in order to evaluate the possible applications of extracts and essential oils, and promote their use in the nutraceutical or food industries. In addition, the antioxidant, hypoglycaemic and hypolipidemic proprieties correlated to chemical composition were studied.

4.4.1 Extraction yields

C. × *clementina* peels extraction yields were reported in Table 22.

Generally, samples obtained by Soxhlet extraction procedure showed the highest yield of extraction with 14.99, 14.42, and 14.28 %, respectively for BO1, BC1, and BR1, respectively.

For extracts obtained by ultrasound assisted maceration process with EtOH/H_2O as solvent, different extractive yields were observed. In fact, BC2 showed a higher yield followed by BO2, while BR2 showed a lower extractive yield (12.91, 11.50, and 9.48 %, respectively). The same trend was also observed for the extract obtained by ultrasound assisted maceration process with EtOH alone with percentage of 12.48, 10.74, and 10.49% for BC5, BO5, and BR5, respectively.

Comparison of data evidenced that higher yields were recorded by using Soxhlet extractor followed by ultrasound assisted maceration process with EtOH/H_2O.

Table 22: Yield extractive of *C.× clementina* peels extracts and essential oils

Sample		Yields (%)
Cetraro		
Soxhlet extractor	BC1	14.42 ± 1.41^{a}
Ultrasuond EtOH/H_2O	BC2	12.91 ± 1.24^{c}
Ultrasuond EtOH	BC3	12.07 ± 1.23^{e}
Maceration EtOH/H_2O	BC4	12.86 ± 1.27^{c}
Maceration EtOH	BC5	12.48 ± 1.20^{d}
Hydrodistillation	BC6	0.54 ± 0.05^{p}
Rosarno		
Soxhlet extractor	BR1	14.28 ± 1.42^{b}
Ultrasuond EtOH/H_2O	BR2	9.48 ± 0.97^{n}
Ultrasuond EtOH	BR3	10.62 ± 1.01^{i}
Maceration EtOH/H_2O	BR4	9.52 ± 0.94^{n}
Maceration EtOH	BR5	10.49 ± 1.00^{l}
Hydrodistillation	BR6	0.80 ± 0.08^{o}
Corigliano Calabro		
Soxhlet extractor	BO1	14.99 ± 1.47^{a}
Ultrasuond EtOH/H_2O	BO2	11.50 ± 1.11^{e}
Ultrasuond EtOH	BO3	10.88 ± 1.04^{g}
Maceration EtOH/H_2O	BO4	9.70 ± 0.93^{m}
Maceration EtOH	BO5	10.74 ± 1.07^{h}
Hydrodistillation	BO6	0.76 ± 0.07^{o}
Sign.		**

Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same column with different small letters differ significantly ($p< 0.05$).

4.4.2 *C.× clementina* peels essential oil analysis

Constituents of *C.× clementina* peels essential oils were listed in order of their elution time on the HP-5 column in Table 23. Twenty constituents were identified in the oils. The extraction of essential oils is the main source of industrial exploitation of *Citrus* products.

The main compound was limonene with a content of 75.11, 85.08 and 61.31%, respectively for BC6, BR6 and BO6, followed by linalool (3.29-6.63%). Limonene is responsible for the taste of *Citrus* fruits while linalool is responsible for the flower odour. Both are used as aromatizes in food products (Buettner et al., 2003).

Interesting, the content in α-pinene was higher in BO6 compared to BC6, while (E)-β-ocimene was present only in the BC6 (3.31%). Additionally, myrcene was presented in percentages 4-times higher for BO6 compared to the other two essential oils. Different concentration of sabinene were also observed with values of 2.55, 0.97 and 1.52%, respectively for BC6, BR6, and BO6, respectively. The different profile supports the hypothesis proposed by Pitarokili et al. (2003) that both exogenous and endogenous factors are able to change the presence/absence or abundance of a single component within the essential oil.

Limonene and linalool were the two main abundant compounds of clementine cv Oroval peels oil from Sicily (Ruberto & Rapisarda, 2002). Bermejo et al. (2011) analysed

the essential oil obtained from three *C.* × *clementina* cv: Fino, Loretina and Marisol peels from Spain. The data obtained showed that, limonene was the most abundant monoterpene followed by the compound myrcene and linalool. This result agreed with Nguyen et al. (2016) in which the main constituents in essential oil, obtained by Vietnam clementine peels, were limonene, myrcene and α-pinene. Recently, Boudries et al. (2017) reported, as main constituents of peels essential oil of Algerian clementine, limonene (96.75%), β-myrcene (2.07%) and sabinene (0.61%).

Table 23. Chemical components of *C.*× *clementina* peels essential oils

Compound	RI[1]	%			I.M[2]	Sign.
		BC6	*BR6*	*BO6*		
α-Pinene	938	1.10 ± 0.12^{c}	1.55 ± 0.21^{b}	3.13 ± 0.33^{a}	1,2,3	**
Sabinene	973	2.55 ± 0.70^{a}	0.97 ± 0.12^{c}	1.52 ± 0.21^{b}	1,2,3	**
β-Pinene	980	*tr*	*tr*	*tr*	1,2,3	**
Myrcene	993	3.56 ± 0.31^{c}	4.94 ± 0.46^{b}	9.10 ± 0.91^{a}	1,2,3	**
dl-Limonene	1030	75.11± 4.55^{b}	85.09 ± 5.12^{a}	61.31 ± 4.02^{c}	1,2,3	**
(*E*)-β-Ocimene	1049	3.31 ± 0.37^{a}	*nd*	*nd*	1,2	**
γ-Terpinene	1057	0.33 ± 0.03^{a}	*tr*	0.32 ± 0.03^{a}	1,2,3	**
Terpinolene	1086	*tr*	*tr*	0.30 ± 0.03^{a}	1,2,3	**
Linalool	1098	5.30 ± 0.55^{b}	3.29 ± 0.36^{c}	6.64 ± 0.61^{a}	1,2,3	**
Nonanale	1100	1.84 ± 0.23^{b}	1.62 ± 0.24^{c}	3.64 ± 0.34^{a}	1,2	**
Citronellal	1148	0.40 ± 0.04^{b}	*tr*	0.75 ± 0.07^{a}	1,2	**
Terpinen-4-ol	1176	0.56 ± 0.05^{b}	0.20 ± 0.02^{c}	0.88 ± 0.08^{a}	1,2	**
α-Terpineol	1189	0.47 ± 0.04^{b}	0.49 ± 0.04^{b}	1.55 ± 0.12^{a}	1,2,3	**
Decanale	1205	1.05 ± 0.10^{b}	1.04 ± 0.10^{b}	2.20 ± 0.21^{a}	1,2	**
trans-Caryophylllene	1415	*tr*	*tr*	*tr*	1,2,3	**
trans-β-Farnesene	1441	*tr*	*tr*	0.35 ± 0.03^{a}	1,2	**
α-Humulene	1454	*tr*	*tr*	*tr*	1,2,3	**
δ-cadinene	1526	0.31 ± 0.02^{a}	0.22 ± 0.02^{c}	0.26 ± 0.02^{b}	1,2	**
β-Sinensal	1697	0.29 ± 0.02^{a}	*tr*	0.13 ± 0.02^{b}	1,2	**
α-Sinensal	1750	0.70 ± 0.07^{a}	0.37 ± 0.04^{c}	0.45 ± 0.05^{b}	1,2	**
Total identified		96.88	99.78	92.53		

Note: Data are reported as mean ± standard deviation (n = 3). [1]Retention indices on the HP 5MS column. [2]M, identification method: 1 – comparison of retention times; 2 – comparison of mass spectra with MS libraries, 3 – comparison with authentic compounds; *tr*: trace (<0.1%). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05).

Previously, Lota et al. (2001) evaluated the chemical composition of clementine essential oil of different cv from Corsica namely MA3, Nules, MA2, Hernandina, Tardia Villareal, Reina, Caffin, MacBean, Oroval, Monreal, Bruno, Tomatera, Commune, Marisol, Ragheb and Guillermina. The following order rank can be observed: limonene> myrcene> linalool. Limonene was quantified in higher percentages (88.1-95.5%), while all the other constituents was quantified in lower percentages. In particular, the linalool was present in a percentage of 0.8-2.3% and myrcene 1.4-2.05. Similar values were found for α-sinensal and β-sinensal. In addition, El-hawary et al. (2013) confirmed that limonene (88.73%) and myrcene (2.97%) represented the main constituents of the essential oil obtained by clementine peels oil from Egypt.

4.4.3 Phytochemicals content in *C.× clementina* peels extracts

The content of bioactive substances in *C.× clementina* peels extracts was analysed. Data were reported in Table 24. The higher total phenol content was observed in extracts obtained by Soxhlet extractor followed by ultrasound assisted maceration process in EtOH/H_2O. In particular, the greatest content was found in BC1 with values of 8.75 mg CAE/g FW followed by BR1 with content of 7.13 mg CAE/g FW. Interesting results, were also observed for BC2 and BC3 with values of 6.30 and 6.27 CAE/g FW, respectively. Analysis of data obtained by maceration extraction procedure with EtOH/H_2O and EtOH as solvent system evidenced that a TPC in the range 3.45-5.34 mg CAE/g FW and a TFC in the range 5.85-5.49 mg CAE/g FW, respectively were observed.

Our data disagree with those reported by Casacchia et al. (2019) that found higher values for both TPC and TFC. Boudries et al. (2015) evaluate the TPC in Algerian clementine peels extracts and found values ranging from 9686.2 to 11934.5 mg GAE/100g DW with a rank of order: cv Cadoux> Monreal> St Martin> Merme> Rocamora> Cheylard.

Table 24. Phytochemicals content of *C.× clementina* peels extracts.

Sample	Total phenols Content[1]	Total flavonoids Content[2]	Total carotenoids Content[3]
BC1	8.75 ± 0.83^{a}	6.05 ± 0.67^{a}	39.84 ± 3.47^{a}
BC2	6.30 ± 0.64^{c}	4.02 ± 0.42^{d}	17.89 ± 1.76^{c}
BC3	6.27 ± 0.68^{c}	5.08 ± 0.58^{b}	16.66 ± 1.63^{d}
BC4	5.34 ± 0.56^{f}	4.02 ± 0.44^{d}	15.60 ± 1.57^{g}
BC5	5.49 ± 0.51^{e}	3.89 ± 0.46^{e}	12.14 ± 1.25^{i}
BR1	7.13 ± 0.78^{b}	4.64 ± 0.47^{c}	19.62 ± 1.98^{b}
BR2	4.43 ± 0.45^{h}	3.13 ± 0.33^{l}	10.88 ± 1.11^{m}
BR3	4.27 ± 0.43^{l}	3.39 ± 0.31^{h}	10.42 ± 1.05^{o}
BR4	4.38 ± 0.44^{i}	3.44 ± 0.36^{h}	15.97 ± 1.69^{f}
BR5	3.99 ± 0.45^{m}	3.20 ± 0.34^{i}	11.68 ± 1.11^{l}
BO1	5.91 ± 0.56^{d}	4.62 ± 0.49^{c}	16.49 ± 1.68^{e}
BO2	4.50 ± 0.41^{g}	3.56 ± 0.34^{g}	9.66 ± 0.92^{q}
BO3	3.80 ± 0.38^{o}	3.80 ± 0.36^{f}	10.28 ± 1.05^{p}
BO4	3.45 ± 0.36^{p}	2.47 ± 0.22^{n}	10.48 ± 1.02^{n}
BO5	3.85 ± 0.37^{n}	2.78 ± 0.28^{m}	13.60 ± 1.37^{h}
Sign.	**	**	**

Data represent means ± SD (standard deviation) (n = 3). [1]mg Chlorogenic Acid Equivalent (CAE)/g fresh weight (FW); [2]mg of Quercetin Equivalents (QE)/g FW. [3]mg β-carotene equivalents/g FW. Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same column with different small letters differ significantly ($p< 0.05$). Sign: significant.

Comparison with our data with literature revealed that *C. × clementine* cv Cadoux and Merme peels extracts from Algeria were richest in TFC with values of 1.047.2 and 942.5 mg EC/100g DW, respectively (Boudries et al., 2015). Additionally, Levaj et al. (2009) showed a TFC of 804.26 mg/100g DW in clementine peels collected in Algeria.

Regarding *C. × clementina* TCC, Soxhlet extraction procedure was the most effective to enrich extract with this class of phytochemical (BC1, 39.84 mg equivalent β-

carotene/g FW). Cetraro samples obtained by ultrasound assisted maceration process in EtOH/H_2O and EtOH also showed a promising TCC content with values of 17.89 and 16.66 mg equivalent β-carotene/g FW, respectively in comparison to Rosarno samples (10.42 and 10.88 mg equivalent β-carotene/g FW, respectively) and Corigliano Calabro samples (10.28 and 9.66 mg equivalent β-carotene/g FW, respectively) obtained with the same extraction procedure.

A lower TCC was reported by Boudries et al. (2015) in Algerian *C.* × *clementina* cv Rocamora, cv Cadoux and St Martin with values of 76.0, 72.9 and 70.0 mg/100g DW, respectively.

4.4.4 HPLC analysis of *C.* × *clementina* peels extracts

Phenolic profile

The HPLC-DAD phenolic profile of peels extracts was reported in Tables 25, 26, and 27. Caffeic acid, eriocitrin, neoeritrocin, isoquercetin, quercetin, hesperidin, poncirin, luteolin, sinensetin and tangeretin were selected as markers and quantified.

Hesperidin was the dominant compound with concentration in the range of 100.26-1093.36 mg/100g FW. In addition, notable quantities of sinensetin (19.56-37.09 mg/100g FW), tangeretin (5.43-8.31 mg/100g FW) and luteolin (3.02-8.36 mg/100g FW) were observed. Interesting, neoeritrocin was absent in samples collected in Rosarno and Corigliano Calabro, but present in the range of 0.68-4.89 mg/100g FW in Cetraro samples. Eriocitrin was, also, absent in Rosarno extracts, but presented in low concentrations in BO3 and BO5 and in a range of 1.56-3.75 mg/100g FW in Cetraro samples.

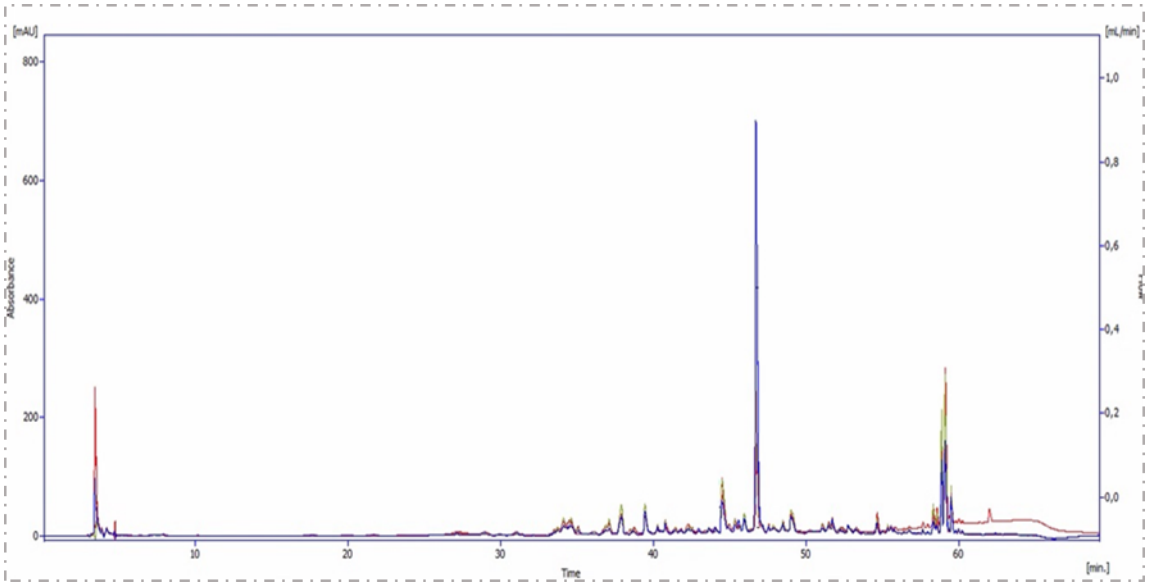


Fig.16. BR3 phenolic profile

Table 25. HPLC-DAD of *C.× clementina* peels extract from Cetraro (mg/100g FW)

Markers	BC1	BC2	BC3	BC4	BC5	Sign.
Caffeic acid	0.22 ± 0.02^{b}	6.98 ± 0.64^{a}	0.15 ± 0.01^{c}	6.40 ± 0.61^{a}	0.20 ± 0.02^{b}	**
Eriocitrin	3.75 ± 0.33^{a}	2.12 ± 0.21^{b}	1.56 ± 0.14^{c}	2.02 ± 0.26^{b}	3.62 ± 0.36^{a}	**
Neoeriocitrin	4.89 ± 0.57^{a}	3.29 ± 0.32^{b}	2.43 ± 0.26^{d}	2.99 ± 0.37^{c}	0.68 ± 0.077^{e}	**
Hesperidin	778.70 ± 15.91^{d}	1007.86 ± 18.12^{b}	1093.36 ± 18.13^{a}	228.63 ± 8.13^{l}	238.53 ± 8.22^{i}	**
Poncirin	1.98 ± 0.12^{b}	0.57 ± 0.05^{c}	2.29 ± 0.34^{a}	2.53 ± 0.28^{a}	2.65 ± 0.02^{a}	**
Isoquercetin	11.26 ± 1.27^{a}	8.49 ± 0.96^{b}	6.61 ± 0.68^{d}	8.20 ± 0.83^{c}	8.45 ± 0.08^{b}	**
Quercetin	8.98 ± 0.93^{a}	7.98 ± 0.72^{b}	6.22 ± 0.61^{d}	7.72 ± 0.77^{c}	7.95 ± 0.08^{b}	**
Luteolin	6.17 ± 0.68^{a}	5.16 ± 0.50^{bc}	4.05 ± 0.47^{d}	5.02 ± 0.54^{bc}	5.39 ± 0.05^{b}	**
Sinensetin	32.86 ± 3.23^{a}	29.36 ± 2.94^{b}	27.07 ± 2.76^{c}	26.22 ± 2.62^{d}	32.56 ± 3.21^{a}	**
Tangeretin	7.47 ± 0.78^{b}	7.92 ± 0.80^{a}	6.57 ± 0.77^{c}	5.43 ± 0.53^{d}	7.19 ± 0.79^{b}	**

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same row with different small letters differ significantly ($p< 0.05$). Sign. significant

Table 26. HPLC-DAD of *C.× clementina* peels extract from Rosarno (mg/100g FW)

Markers	BR1	BR2	BR3	BR4	BR5	Sign.
Caffeic acid	*nd*	5.32 ± 0.51^{c}	*nd*	8.99 ± 0.94^{b}	10.87 ± 1.22^{a}	**
Eriocitrin	*nd*	*nd*	*nd*	*nd*	*nd*	**
Neoeriocitrin	*nd*	*nd*	*nd*	*nd*	*nd*	
Hesperidin	977.23 ± 18.1^{a}	558.14 ± 15.24^{c}	667.18 ± 15.54^{b}	100.26 ± 8.15^{e}	173.52 ± 8.13^{d}	**
Poncirin	1.33 ± 0.17^{b}	1.35 ± 0.18^{b}	1.39 ± 0.17^{a}	*nd*	*n.d*	**
Isoquercetin	7.29 ± 0.71^{a}	7.27 ± 0.73^{a}	*nd*	*nd*	*nd*	**
Quercetin	6.86 ± 0.64^{b}	8.02 ± 0.84^{a}	5.78 ± 0.52^{c}	*nd*	*nd*	**
Luteolin	5.53 ± 0.53^{c}	3.77 ± 0.48^{d}	3.53 ± 0.39^{de}	8.2 ± 0.87^{a}	5.74 ± 0.57^{b}	**
Sinensetin	37.99 ± 3.74^{a}	25.59 ± 2.11^{e}	26.39 ± 2.63^{d}	37.2 ± 3.40^{b}	31.41 ± 3.12^{c}	**
Tangeretin	8.31 ± 0.85^{b}	6.47 ± 0.66^{e}	7.0 ± 0.77^{d}	9.6 ± 0.97^{a}	7.37 ± 0.76^{c}	**

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p< 0.05$. Means in the same row with different small letters differ significantly ($p< 0.05$). *nd*: not detected. Sign: significant.

Table 27. HPLC-DAD of *C.× clementina* peels extract from Corigliano Calabro (mg/100g FW)

Marcatori selezionati	BO1	BO2	BO3	BO4	BO5	Sign.
Caffeic acid	2.68 ± 0.31^{b}	8.0 ± 0.82^{a}	*nd*	*nd*	*nd*	**
Eriocitrin	*nd*	0.73 ± 0.07^{b}	0.88 ± 0.08^{a}	*nd*	0.78 ± 0.08^{b}	**
Neoeriocitrin	*nd*	*nd*	*nd*	*nd*	*nd*	**
Hesperidin	243.98 ± 10.21^{c}	364.52 ± 11.33^{a}	253.8 ± 10.15^{b}	155.28 ± 8.12^{e}	179.42 ± 8.16^{d}	**
Poncirin	1.51 ± 0.19^{b}	1.21 ± 0.17^{c}	1.61 ± 0.17^{a}	1.08 ± 0.17^{cd}	1.05 ± 0.18^{cd}	**
Isoquercetin	7.58 ± 0.71^{c}	6.37 ± 0.66^{e}	6.66 ± 0.63^{d}	10.54 ± 1.26^{a}	9.65 ± 0.92^{b}	**
Quercetin	7.13 ± 0.77^{b}	7.76 ± 0.88^{a}	7.7 ± 0.87^{a}	7.07 ± 0.72^{b}	6.51 ± 0.65^{c}	**
Luteolin	6.11 ± 0.63^{c}	4.72 ± 0.43^{d}	3.02 ± 0.32^{e}	8.58 ± 0.94^{a}	8.36 ± 0.87^{b}	**
Sinensetin	31.51 ± 3.14^{a}	30.74 ± 3.07^{b}	19.56 ± 2.19^{e}	25.7 ± 2.56^{c}	21.2 ± 2.13^{d}	**
Tangeretin	6.75 ± 0.62^{a}	6.06 ± 0.61^{b}	6.2 ± 0.63^{bc}	5.8 ± 0.68^{c}	5.55 ± 5.01^{l}	**

Data represent means ± SD (standard deviation) ($n = 3$). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** $p < 0.05$. Means in the same column with different small letters differ significantly ($p < 0.05$). *nd*: not detected. Sign: significant.

A great difference was found also regarding caffeic acid concentration that was contained in concentration of 10.87 and 8.99 mg/100g FW in both BR5 and BR4, respectively, but it was absent in Corigliano Calabro extracts obtained with the same extraction technique. The contents of isoquercetin and quercetin were, also, reported. In particular, both flavonoids were absent in both BR4 and BR5, while, notable contents were reported for other extracts (6.37-11.26 mg/100g FW for isoquercetin and 5.78-8.89 mg/100g FW for quercetin).

Bermejo et al. (2011) reported the phenolic profile of *C.* × *clementina* peels cv Fino, Loretina and Marisol collected in Spain. Hesperidin was found in concentration ranging from 33.5 to 38.64 mg/g DW whereas sinensetin in the range 0.15-0.25 mg/g of DW. Values of 0.27, 0.69 and 0.37 mg/g DW were found for tangeretin in cv Fino, Loretina and Marisol, respectively. A lower content of hesperidin (47.22 mg/100g DW) was found in clementine peels extract from Corsica (Levaj et al., 2009). The high amount of hesperidin in clementine peels was confirmed, also, by Hayat et al. (2016) and Tumbas et al. (2010) with values in the range 0.39-15.3 mg/g DW.

These results agree with our data and with those reported by Nogata et al. (2006), in which the most abundant constituent of clementine peels was hesperidin (1800 mg/100g FW) followed by narirutin (57.8 mg/100g FW) and diosmin (35.4 mg/100g FW), latter not selected by us as markers.

Therefore, it emerged that the different chemical composition of *C.* × *clementina* peels is subject to changes under the influence of various factors including cultivars, climatic conditions, environmental factors and fruit ripening phase (M'hiri et al., 2016).

Coumarins determination

The obtained data excluded the presence of furanocoumarins in all investigated samples.

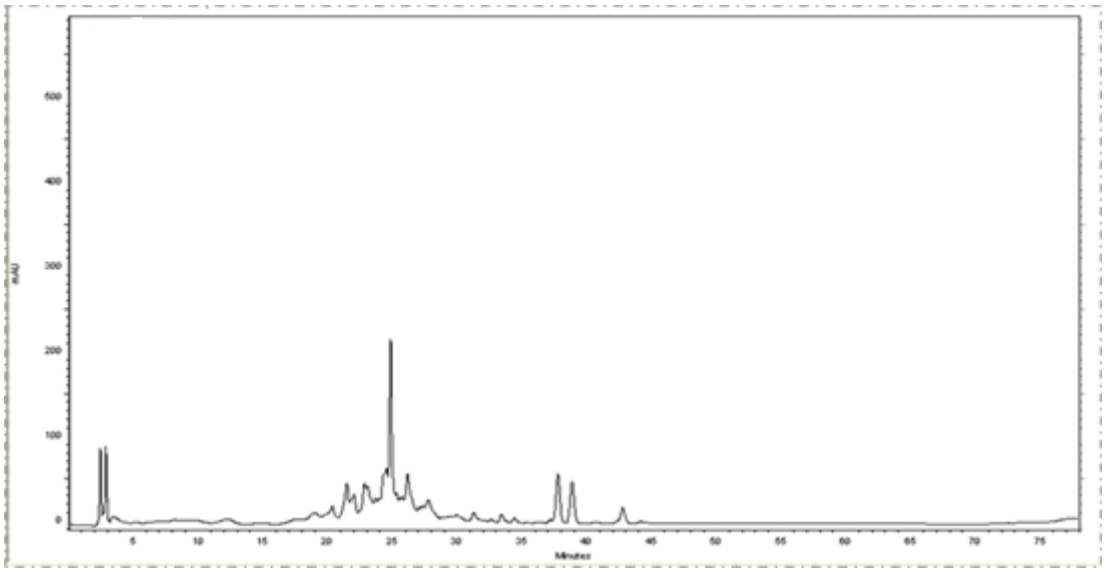


Fig. 17. Coumarins determination in BR3

Bourgaud et al. (2006) reported a content in clementine peels extract of isopimpinellin and bergapten of 1.40 and 0.96 mg/kg of FW, respectively. Bergamottin was found in traces (Melough et al., 2017; Bourgaud et al., 2006). More recently, Ramírez-Pelayo et al. (2019) investigated the presence of coumarins and furanocoumarins in peels from *C. sinensis* var. Valencia, *C. reticulata* var. Arrayana and Oneco, *C. aurantifolia* var. Pajarito, *C.* × *limonia* and *C. latifolia*. The coumarins profile dependent from the species.

4.4.5 Antioxidant activity

The antioxidant activity of *C.* × *clementina* peels extracts were showed in Table 28. All tested samples showed antioxidant activity in a concentration-dependent manner.

The greatest antioxidant potential was found with Cetraro samples in DPPH test. In particular, BC3 showed an IC_{50} value of 45.79 μg/mL followed by BC2 (IC_{50} value of 52.58 μg/mL). Interesting results were also obtained with BC2 and BR1 with IC_{50} values of 54.58 and 68.13 μg/mL, respectively. In the ABTS test, both BC2 and BO5 showed the lowest IC_{50} value of 8.22 μg/mL followed by BR3 (IC_{50} value of 9.47 μg/mL).

All samples obtained by Soxhlet extractor exhibited a greater ability to inhibit lipid peroxidation after 30 min of incubation. While, after 60 minutes of incubation, the best results can be attributed to samples collected in Rosarno and Cetraro and obtained by ultrasound assisted maceration process in EtOH (IC_{50} values of 8.78 and 16.47 μg/mL, respectively).

In the FRAP test, BR3 showed a greater ability to reduce iron ions (54.95 μM Fe (II)/mg), followed by BR4 and BC3 (40.46 and 34.28 μM Fe (II)/mg, respectively).

As showed in Table 29, the most significant correlation was found between caffeic acid and β-carotene bleaching test after 30 min incubation (r = 0.78).

Concerning essential oils activity, several differences were displayed in β-carotene bleaching test after 30 min of incubation. In fact, both BR6 and BO6 were more active than CE6. The same observation could be done in FRAP test in which BO6 and BR6 presented the higher activity. No differences were recorded in radical scavenging activity data evaluated by DPPH and ABTS tests.

Limonene, the main abundant compounds in *C.* × *clementina* essential oils, was positively correlated with ABTS test (r = 0.99) (Table 30). While, sabinene, (*E*)-β-Ocimene, β-sinensal and α-sinensal presented a positive correlation with β-carotene bleaching test (t= 30 min) with r values of 1.00, 1.00, 0.99 and 0.98, respectively. The same compounds, was positively correlated, also, with β-carotene bleaching test (t=60 min) with r values of 1.00, 0.99, 1.00 and 1.00, respectively.

Table 28. Antioxidant activity *in vitro* of *C.× clementina* peels samples.

Sample	DPPH test (IC_{50} µg/mL)	ABTS test (IC_{50} µg/mL)	β-carotene bleaching test t 30 min (IC_{50} µg/mL)	β-carotene bleaching test t 60 min (IC_{50} µg/mL)	FRAP µM Fe (II)/g
BC1	$105.66 \pm 4.01^{****}$	$14.82 \pm 1.22^{****}$	$11.22 \pm 1.08^{****}$	$32.52 \pm 1.93^{****}$	$23.51 \pm 1.77^{****}$
BC2	$52.58 \pm 2.32^{****}$	$8.22 \pm 0.84^{***}$	$22.86 \pm 1.93^{****}$	$75.03 \pm 2.77^{****}$	$28.48 \pm 1.83^{****}$
BC3	$45.79 \pm 2.14^{****}$	$15.06 \pm 1.62^{****}$	$12.53 \pm 1.17^{****}$	$16.47 \pm 1.85^{****}$	$34.28 \pm 1.95^{****}$
BC4	$117.86 \pm 4.07^{****}$	$17.54 \pm 1.91^{****}$	$12.53 \pm 1.26^{****}$	$90.99 \pm 3.93^{****}$	$26.26 \pm 1.77^{****}$
BC5	$140.39 \pm 4.35^{****}$	$21.03 \pm 1.79^{****}$	$10.47 \pm 1.08^{****}$	$18.26 \pm 1.81^{****}$	$23.19 \pm 1.63^{****}$
BC6	$308.55 \pm 6.12^{****}$	$24.13 \pm 2.03^{****}$	$47.22 \pm 2.21^{****}$	$91.92 \pm 3.98^{****}$	$6.13 \pm 0.61^{****}$
BR1	$68.13 \pm 2.43^{****}$	$15.21 \pm 1.23^{****}$	$11.45 \pm 1.16^{****}$	$36.84 \pm 1.94^{****}$	$30.97 \pm 1.97^{****}$
BR2	$81.26 \pm 3.41^{****}$	$10.97 \pm 1.05^{****}$	$58.52 \pm 2.57^{****}$	$68.75 \pm 2.96^{****}$	$25.78 \pm 1.73^{****}$
BR3	$113.17 \pm 4.03^{****}$	$9.47 \pm 0.97^{****}$	$19.38 \pm 1.83^{****}$	$8.78 \pm 0.83^{****}$	54.95 ± 2.14^{ns}
BR4	$83.14 \pm 3.61^{****}$	$20.25 \pm 1.94^{****}$	$89.39 \pm 3.82^{****}$	$96.8 \pm 3.18^{****}$	$40.46 \pm 2.47^{****}$
BR5	$125.37 \pm 4.11^{****}$	$11.76 \pm 1.13^{****}$	$72.4 \pm 2.71^{****}$	$43.22 \pm 2.11^{****}$	$21.13 \pm 1.03^{****}$
BR6	$370.3 \pm 6.74^{****}$	$26.30 \pm 2.07^{****}$	$10.37 \pm 0.93^{****}$	$55.7 \pm 2.53^{****}$	$23.91 \pm 1.74^{****}$
BO1	$259.57 \pm 5.31^{****}$	$31.50 \pm 2.11^{****}$	$11.41 \pm 0.94^{****}$	$71.00 \pm 2.64^{****}$	$6.48 \pm 0.62^{****}$
BO2	$174.17 \pm 4.43^{****}$	$28.37 \pm 2.07^{****}$	$64.15 \pm 2.76^{****}$	$72.78 \pm 2.75^{****}$	$21.26 \pm 1.71^{****}$
BO3	$212.65 \pm 3.83^{****}$	$15.21 \pm 1.13^{****}$	$39.09 \pm 2.17^{****}$	$87.98 \pm 2.83^{****}$	$27.39 \pm 1.37^{****}$
BO4	$169.48 \pm 4.87^{****}$	$14.05 \pm 1.25^{****}$	$39.09 \pm 2.16^{****}$	$87.98 \pm 3.61^{****}$	$26.1 \pm 1.83^{****}$
BO5	$212.65 \pm 4.52^{****}$	$8.22 \pm 0.89^{***}$	$12.90 \pm 0.95^{****}$	$58.33 \pm 2.59^{****}$	$27.39 \pm 1.85^{****}$
BO6	$333.7 \pm 6.01^{****}$	$18.31 \pm 1.91^{****}$	$11.78 \pm 1.02^{****}$	$61.8 \pm 2.62^{****}$	$25.38 \pm 1.71^{****}$
Positive control					
Ascorbic acid	5.0 ± 0.8	1.7 ± 0.06			
Propyl gallate			0.09 ± 0.004	$0.0\ 9 \pm 0.004$	
BHT					63.2 ± 2.3

Data are expressed as means ± S.D. (n= 3). DPPH Radical Scavenging Activity Assay; Antioxidant Capacity Determined by Radical Cation ($ABTS^{+}$); β-carotene bleaching test; Ferric Reducing Antioxidant Power (FRAP). Ascorbic acid, BHT and Propyl gallate were used as positive control in antioxidant tests. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the positive controls. ns: not significant.

Table 29. *Pearson*'s correlation between phenolic compounds in investigated samples and activity

	DPPH test	ABTS test	β-carotene bleaching test t 30 min	β-carotene bleaching test t 60 min	FRAP test
TPC	0.10				
TFC		0.25			
Caffeic acid		0.25	0.78	0.22	0.13
Quercetin					
Isoquercetin	0.21	0.02		0.14	
Hesperidin					0.16
Luteolin	0.34		0.14	0.17	
Poncirin	0.04	0.20			0.13
Sinensetin		0.19	0.24		
Tangeretin			0.27		0.08

Table 30 *Pearson*'s correlation between EO constituents and activity

	DPPH test	ABTS test	β-carotene bleaching test t 30 min	β-carotene bleaching test t 60 min	FRAP test
α-Pinene	0.10				0.72
Sabinene		0.18	1.00	1.00	
Myrcene					0.74
Octanal	0.13				0.47
Limonene	0.51	0.99		0.06	
(*E*)-β-Ocimene		0.26	1.00	0.99	
γ-Terpinene			0.56	0.66	
Terpinolene					0.56
Linalool			0.15	0.27	
Terpinen-4-ol					0.57
Decanal					0.56
β-Sinensal		0.10	0.99	1.00	
α-Sinensal		0.05	0.98	1.00	

Previously, Loizzo et al. (2012) investigated the antioxidant potential of *C. aurantifolia* peels methanol and *n*-hexane extracts. Methanol extract showed a promising DPPH radical scavenging activity with IC_{50} value of 78.3 μg/mL. Promising results were obtained on protection of lipid peroxidation by using the same extract (IC_{50} values of 25.5 and 36.4 μg/mL for 30 and 60 min incubation, respectively). Values of 18.7 TEAC for ABTS and 146.0 μM Fe(II)/g for FRAP. The results obtained for *n*-hexane extract showed IC_{50} of 131.1 μg/mL for DPPH, 9.7 and 18.5 μg/mL for β-carotene bleaching test after 30 and 60 min of incubation, values of 36.2 TEAC for ABTS and 171.6 μM Fe(II)/g for FRAP. HPLC analysis revealed the presence of poncirin, neoeriocitrin and hesperidin in the MeOH extract. All these compounds are able to inhibit $DPPH^{\cdot}$ radical with IC_{50} values of 44.23, 2.85 and 16.54 μM, respectively. Moreover, neoeriocitrin and hesperidin showed IC_{50} values of 3.18 and 10.81μM, respectively in β-carotene bleaching test (Tundis et al., 2016a).

RACI was used to extrapolate sample with the highest antioxidant potential. Generally, extracts obtained by clementine fruits collected in Cetraro and Rosarno

presented greatest antioxidant potential. In particular, as showed in Figure 18, samples BC3 and BC1 are noteworthy. A promising antioxidant activity was found also for BR3 and BR1.

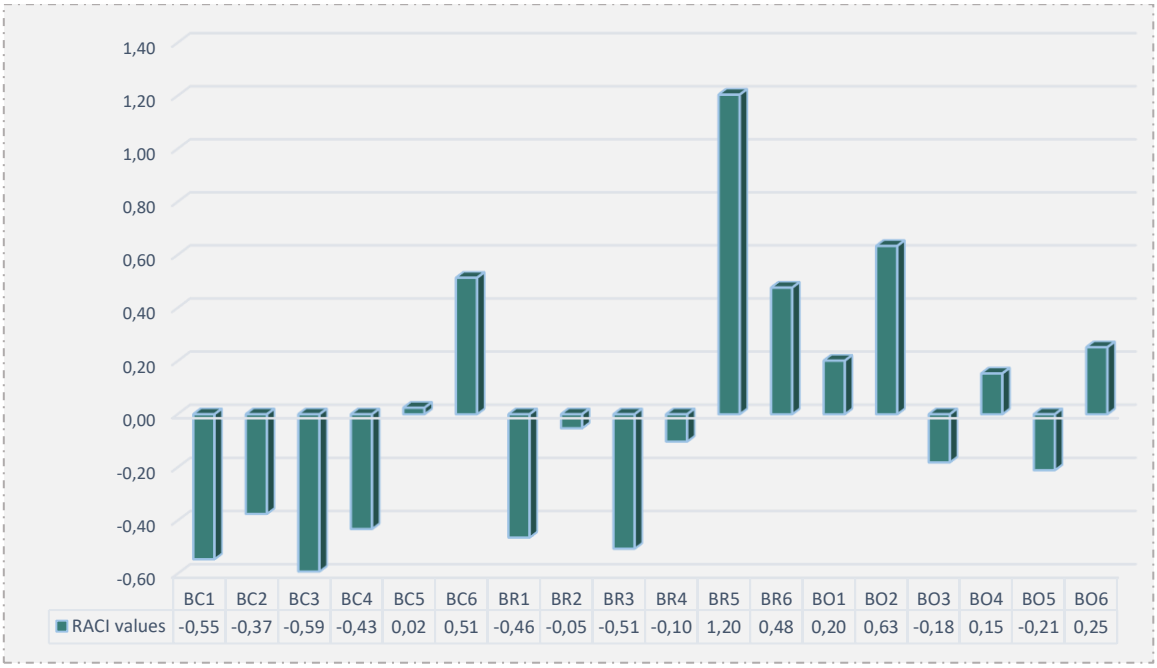


Figure 18. RACI value of *C.* × *clementina* peels extracts

4.4.6 Hypoglycaemic and hypolipidemic activity of *C.* × *clementina* peels extract

The hypoglycaemic activity of the extracts obtained from *C.* × *clementina* peels was evaluated against carbohydrate hydrolysing enzymes α-amylase and α-glucosidase, while hypolipidemic activity against lipase enzyme.

All investigated samples showed inhibitory activity in a concentration-dependent manner (Table 31). Cetraro extract obtained by ultrasound assisted maceration process with EtOH (BC3) showed the highest inhibitory activity with IC_{50} values of 71.79 and 79.73 μg/mL, against α-glucosidase and α-amylase, respectively. Interesting results were, also, observed against α-glucosidase for BC4 (IC_{50} value of 101.91μg/mL) and, for BR4 against α-amylase (IC_{50} of 128.5 μg/mL). This extract was characterized by a considerable tangeretin content, probably responsible of the enzyme's inhibitor effect.

Generally, extracts obtained by ultrasound assisted maceration procedure in both EtOH and EtOH/H_2O showed a greater activity against lipase, except for Rosarno extracts. A promising hypolipidemic potential was found, also, for BC3 that showed an IC_{50} value of 112.0.6 μg/mL against lipase followed by BO3 (IC_{50} value of 132.37 μg/mL).

Table 31. Enzyme inhibitory activity of *C.× clementina* peels samples

Sample	**α-Amylase**	**α-Glucosidase**	**Lipase**
	IC_{50} (μg/ml)	IC_{50} (μg/ml)	IC_{50} (μg/ml)
BC1	210.68 ± 4.9****	141.32 ± 4.3****	186.14 ± 4.2****
BC2	132.00 ± 4.2****	152.15 ± 4.4****	145.59 ± 3.7****
BC3	79.73 ± 3.6****	71.97 ± 2.6****	112.06 ± 3.6****
BC4	146.89 ± 4.3****	101.91 ± 3.9****	186.54 ± 4.2****
BC5	258.13 ± 5.1****	126.75 ± 4.1****	189.37 ± 4.3****
BC6	228.35 ± 4.9****	225.35 ± 4.9****	181.48 ± 4.0****
BR1	154.77 ± 4.4****	152.15 ± 4.4****	171.12 ± 3.8****
BR2	146.02 ± 4.3****	202.07 ± 4.8****	181.37 ± 4.0****
BR3	237.98 ± 4.9****	239.73 ± 5.1****	191.91 ± 4.8****
BR4	128.5 ± 4.1****	256.07 ± 5.2****	174.15 ± 3.8****
BR5	181.93 ± 4.3****	197.69 ± 4.8****	179.63 ± 3.9****
BR6	185.43 ± 4.3****	287.91 ± 5.2****	182.74± 4.1****
BO1	207.33 ± 4.8****	130.25 ± 4.2****	173.42 ± 40****
BO2	167.91 ± 4.5****	129.37 ± 4.2****	142.06 ± 4.4****
BO3	160.91 ± 4.5****	143.39 ± 4.4****	132.37 ± 4.4****
BO4	280.03 ± 5.2****	138.13 ± 4.3***	165.18 ± 3.9***
BO5	186.13 ± 4.6****	224.84 ± 2.9****	179.83 ± 4.0***
BO6	252.00 ± 5.1****	260.76 ± 5.1****	198.36 ± 4.8***
Positive Control			
Acarbose	50.0 ± 0.9	35.5 ± 1.2	
Orlistat			37.5 ± 0.92

Data are expressed as means ± S.D. (n= 3). Acarbose used as positive control in α-amylase and α-glucosidase tests. Orlistat used as positive control in lipase test. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, compared with the positive control.

Mendoza-Sánchez et al. (2019) reported the possible interaction between hesperidin and pancreatic lipase enzyme active site (Figure 19). The main interactions of hesperidin with lipase involved conventional hydrogen bonds with residues Asn^{229}, Cys^{237}, Glu^{37}0, Arg^{384}, Asp^{387} and hydrophobic bond with Tyr369.

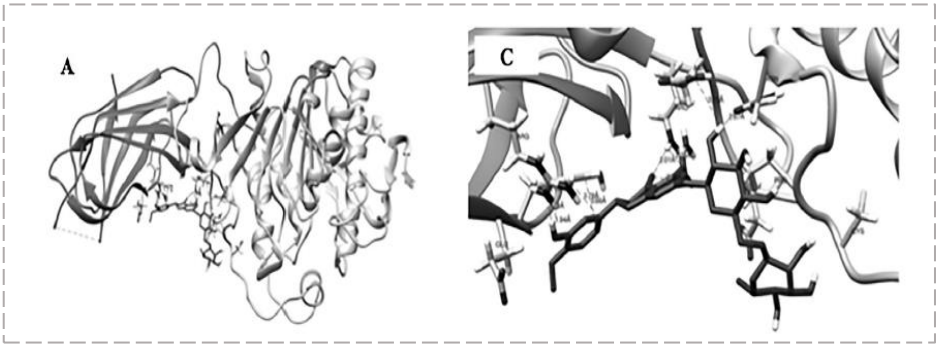


Figure 19. Molecular docking between hesperidin and pancreatic lipase
(Adapted from Mendoza-Sánchez et al., 2019)

4.4.7 PCA peels and essential oils

Principal component analysis (PCA) applied to the data explained 71% of the total variance (Figure 20). The first component (PC1) explains 46% of all information, and separates the extracts which have the highest levels of poncirin, quercetin, isoquercetin and eriocitrin (BC4 and BC5).

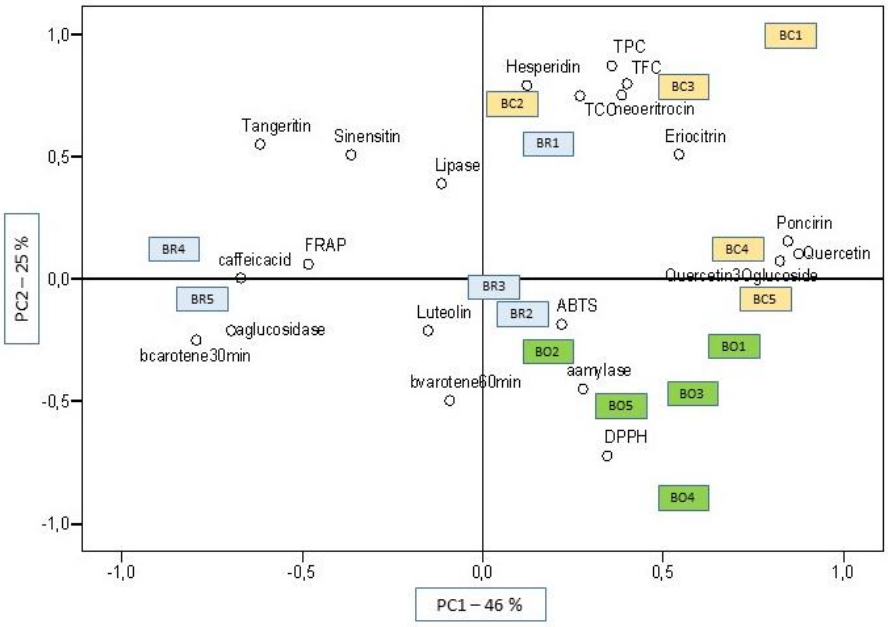


Fig. 20. Graphical representation of scores (BR, BC and BO) and loads (variables) from analyses of fractions of peels, evaluated in relation to the axes defined by the principal components (PC1 and PC2).

The second component (PC2) that discriminates BC1, BC2 and BC3 by the levels TPC, TFC, TCC, hesperidin, neoeriocitrin, sinensetin, tangeretin and lipase, explain the 25% of the total variance. It is noteworthy that Cetraro extracts was separated by the other extract probably due the high content in bioactive compounds.

4.5 *C.* × *clementina* enriched juice

Based on obtained data JF sample, obtained from fruits collected in Corigliano Calabro, was chosen as matrix to be enriched with *C.* × *clementina* by-products (leaves and peels extracts) for the development of functional drink (Table 32).

Table 32. Concentration used for *C.* × *clementine* juice enrichment

Sample	%
JF	Juice control (without extract)
JFA	JF + 20% CO2
JFB	JF + 15% CO2
JFC	JF + 10% CO2
JFD	JF + 5% CO2
JFE	JF + 20% BC3
JFF	JF + 15% BC3
JFG	JF + 10% BC3
JFH	JF + 5% BC3
JFI	JF + 20% CO2 and BC3 (1:1)
JFL	JF + 15% CO2 and BC3 (1:1)
JFM	JF + 10% CO2 and BC3 (1:1)
JFN	JF + 5% CO2 and BC3 (1:1)

For this purpose, CO2 and BC3 samples were added a different concentration (20, 15, 10, and 5% w/v) to the clementine juice (JF). Both extracts were rich in phenolic compounds that were responsible of the significant increase in biological activity of the juices, proportionally to the added concentrations.

The toxicity of flavonoids is very low in animals. For rats, the LD_{50} is 2-10 g per animal for most flavonoids. Similar doses in humans are quite unrealistic. Compared with literature data, it was found that phenolic acids are within the limits of toxicity from medium to weakly toxic (Bose et al., 2018).

As expected the highest antioxidants, hypoglycaemic and hypolipidemic activities were observed at the maximum concentration tested (20% w/v). Moreover, sensory analysis was done in order to verify consumer acceptance.

4.5.1 *C.* × *clementina* enriched juice quality characteristics

The *C.* × *clementina* enriched juice parameters evaluated in this study including TSS, pH, acidity and colour were investigated (Table 33).

The enrichment with *Citrus* by-products did not resulted in significant changes compared to control juice. Minimal variations were observed in the colorimetric

measurement. The enrichment with peels extracts showed similar values to the control juice, while the enrichment with leaves extract gave the juice less bright.

Table 33. Enriched juice quality parameters

Parameters	°Brix	pH	Acidity (g/100mL)	Croma value (C*)	Hue angle in degrees	Hue Angle in radians
JF	10.24 ± 1.03^{a}	3.72 ± 0.07^{a}	0.70 ± 0.02^{bcd}	34.04 ± 2.02^{b}	76.06 ± 2.50^{a}	1.33 ± 0.02^{a}
JFA	10.23 ± 1.14^{a}	3.62 ± 0.04^{f}	0.71 ± 0.03^{a}	33.71 ± 2.00^{f}	72.54 ± 2.41^{b}	1.26 ± 0.02^{a}
JFB	10.22 ± 1.31^{a}	3.64 ± 0.06^{f}	0.71 ± 0.02^{ab}	33.82 ± 2.03^{ef}	72.44 ± 2.42^{b}	1.26 ± 0.01^{a}
JFC	10.22 ± 1.02^{a}	3.68 ± 0.05^{c}	0.70 ± 0.02^{abc}	33.94 ± 2.02^{cde}	72.33 ± 2.43^{b}	1.26 ± 0.03^{a}
JFD	10.22 ± 1.04^{a}	3.7 ± 0.06^{ab}	0.71 ± 0.04^{abcd}	34.02 ± 2.10^{b}	72.17 ± 2.39^{b}	1.26 ± 0.04^{a}
JFE	10.25 ± 1.12^{a}	3.65 ± 0.07^{ed}	0.71 ± 0.02^{abcd}	33.94 ± 2.09^{cde}	71.39 ± 2.34^{c}	1.24 ± 0.02^{a}
JFF	10.24 ± 1.03^{a}	3.67 ± 0.05^{cd}	0.69 ± 0.03^{d}	34.01 ± 2.11^{bc}	71.21 ± 2.35^{c}	1.25 ± 0.03^{a}
JFG	10.23 ± 1.13^{a}	3.68 ± 0.07^{bc}	0.69 ± 0.03^{cd}	34.04 ± 2.02^{b}	71.15 ± 2.36^{c}	1.24 ± 0.02^{a}
JFH	10.22 ± 1.20^{a}	3.71 ± 0.03^{a}	0.71 ± 0.04^{abcd}	34.08 ± 2.07^{bc}	71.03 ± 2.33^{c}	1.24 ± 0.02^{a}
JFI	10.24 ± 1.03^{a}	3.68 ± 0.07^{cd}	0.69 ± 0.03^{d}	33.87 ± 2.04^{e}	72.72 ± 2.42^{a}	1.26 ± 0.03^{a}
JFL	10.23 ± 1.03^{a}	3.69 ± 0.05^{bc}	0.69 ± 0.03^{bcd}	33.91 ± 2.06^{de}	72.61 ± 2.43^{b}	1.26 ± 0.04^{a}
JFM	10.22 ± 1.04^{a}	3.70 ± 0.08^{ab}	0.70 ± 0.04^{abcd}	34.97 ± 2.12^{a}	72.53 ± 2.41^{b}	1.26 ± 0.03^{a}
JFN	10.22 ± 1.02^{a}	3.70 ± 0.08^{ab}	0.71 ± 0.04^{bcd}	34.01 ± 2.07^{bc}	72.27 ± 2.42^{b}	1.26 ± 0.02^{a}
Sign.	ns	**	**	**	**	ns

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05). Sign:significant. ns: not significant.

The total phenols, flavonoids, and carotenoids content was evaluated in all enriched juices (Table 34). As expected, enrichment with *Citrus* by-products resulted in an increase of total phytochemicals content compared to the control juice. In particular, the following rank was found regarding TPC: JFA > JFI > JFB.

Table 34. Total phenols, flavonoids, and carotenoids content of samples juice.

Sample	TPC[1]	TFC[2]	TCC[3]
JF	31.12 ± 1.13^{i}	18.23 ± 0.76^{o}	54.65 ± 1.44^{o}
JFA	41.01 ± 1.04^{a}	28.44 ± 1.04^{a}	61.56 ± 1.76^{e}
JFB	40.26 ± 1.16^{b}	26.11 ± 1.15^{c}	59.78 ± 1.43^{f}
JFC	38.55 ± 1.12^{cd}	24.01 ± 1.12^{f}	57.89 ± 1.17^{i}
JFD	34.34 ± 1.12^{g}	20.55 ± 1.01^{i}	56.76 ± 1.26^{m}
JFE	39.10 ± 1.07^{c}	24.34 ± 1.12^{e}	62.45 ± 2.23^{b}
JFF	38.01 ± 1.43^{d}	22.32 ± 1.10^{g}	61.98 ± 1.99^{d}
JFG	37.43 ± 1.12^{e}	20.43 ± 1.28^{l}	58.89 ± 1.11^{g}
JFH	33.21 ± 1.25^{h}	19.78 ± 0.87^{m}	56.55 ± 1.56^{n}
JFI	40.34 ± 1.11^{b}	26.53 ± 1.12^{b}	62.15 ± 1.43^{c}
JFL	39.76 ± 1.12^{b}	25.22 ± 1.15^{d}	61.89 ± 1.32^{a}
JFM	35.31 ± 1.21^{f}	21.32 ± 1.04^{h}	58.23 ± 1.65^{h}
JFN	33.46 ± 1.12^{h}	19.34 ± 0.45^{n}	57.34 ± 1.12^{l}
Sign.	**	**	**

[1]TPC: Total Phenols Content (mg equivalents of chlorogenic acid/100 mL); [2]TFC: Total Flavonoids Content (mg equivalents of quercetin/100 mL); [3]TCC: Total Carotenoids Content (mg equivalents of β-carotene/100 mL). Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. ** p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05). Sign: significant.

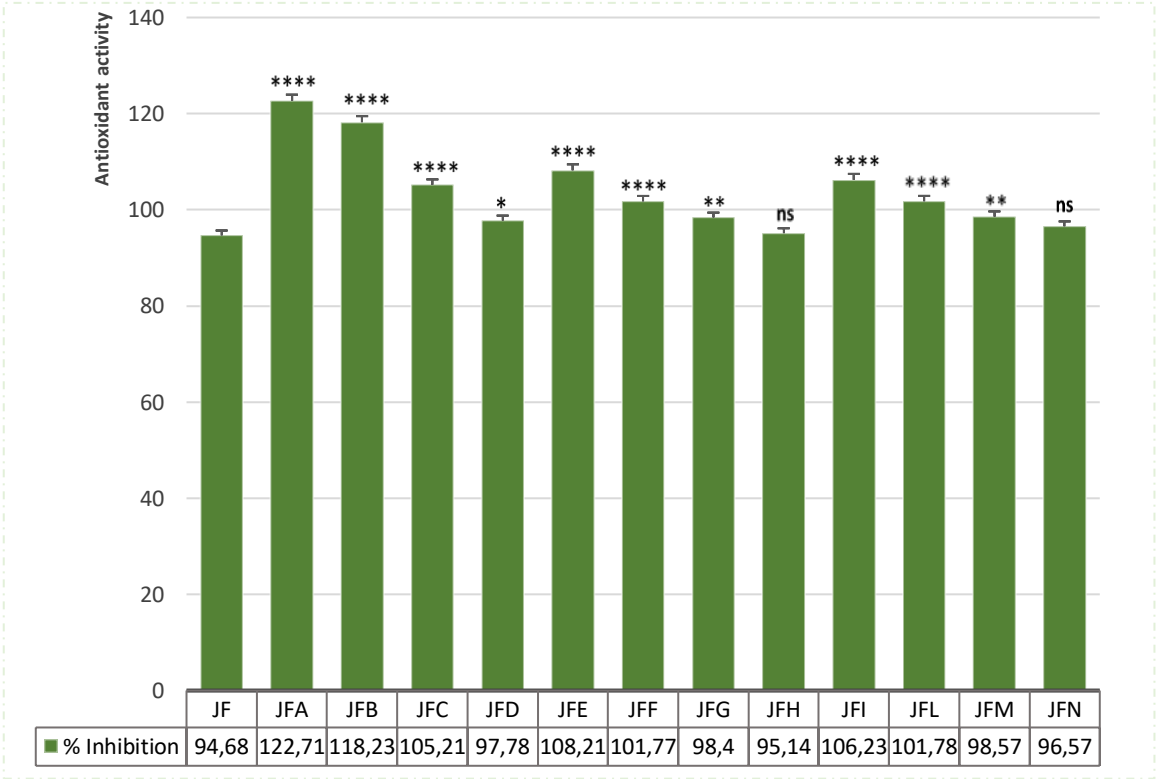
Furthermore, results evidenced that juice enriched with 20% leaves extract had increase the TPC of product by 10% compared to JF. A lower value about 8% was found when JF was enrich with 20% w/v peels extract.

A TFC content ranging from 19.84 to 28.44 mg QE/100 mL was found for JFN and JFA, respectively. Also, in this case, the enrichment with 20% (w/v) of CO2 showed an increase of 10% in the TFC compared with the control (JF). In JFI an increase of 8%, was observed. As observed for TPC the enrichment with 20% (w/v) peels extract showed less increase of TFC value about 6% compared to JF.

No significant differences were recorded in TCC of JFE, JFI and JFD, with values of 62.45, 62.15, and 61.98 mg equivalents of β-carotene/100 mL, respectively. Furthermore, results showed that enrichment with 20 and 15% (w/v) of BC3 cause an increased TCC by 8 and 7 %, respectively compared to control juice. The enrichment with 20% of CO2, showed an increase only of 7 % compared to JF.

4.5.2 Antioxidant activity

Figure 21 showed the DPPH radical scavenging activity of enriched juices. Generally, the increase in antioxidant activity was significantly higher in juices enriched with leaves extracts with percentage of 28% for juice enriched with leaves extract at concentration of 20% w/v.

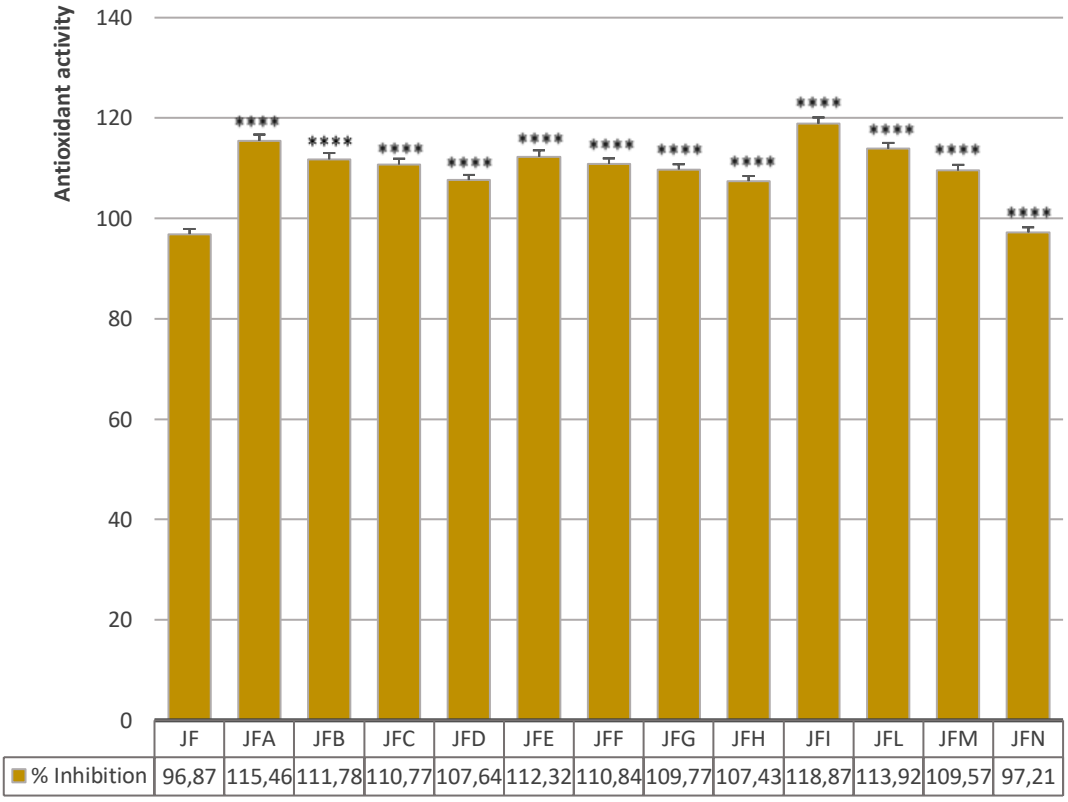


Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p< 0.01, *p< 0.1 compared with the negative controls (control juice). ns: not significant

Fig. 21. DPPH radical scavenging activity of control and enriched juices

An increased radical scavenging potential that reach the 13% was observed when 20% w/v peels extract was added to the juice. A promising result in DPPH test was observed, also, with JFB that increased the antioxidant activity of the juice about 24%.

Figure 22 showed the ABTS results of investigated samples. In this case, the higher ABTS radical scavenging activity was observed for JFI, followed by JFA. Similar value was found, also, for JFE. Juice that received leaves and peels extracts (1:1) at concentration of 20% w/v showed an increase of 22% in antioxidant activity compared to the juice control. For addition of 20% w/v leaves extracts (JFA) and peels extracts (JFE) to the juice an increase of 19 and 15%, respectively was observed.



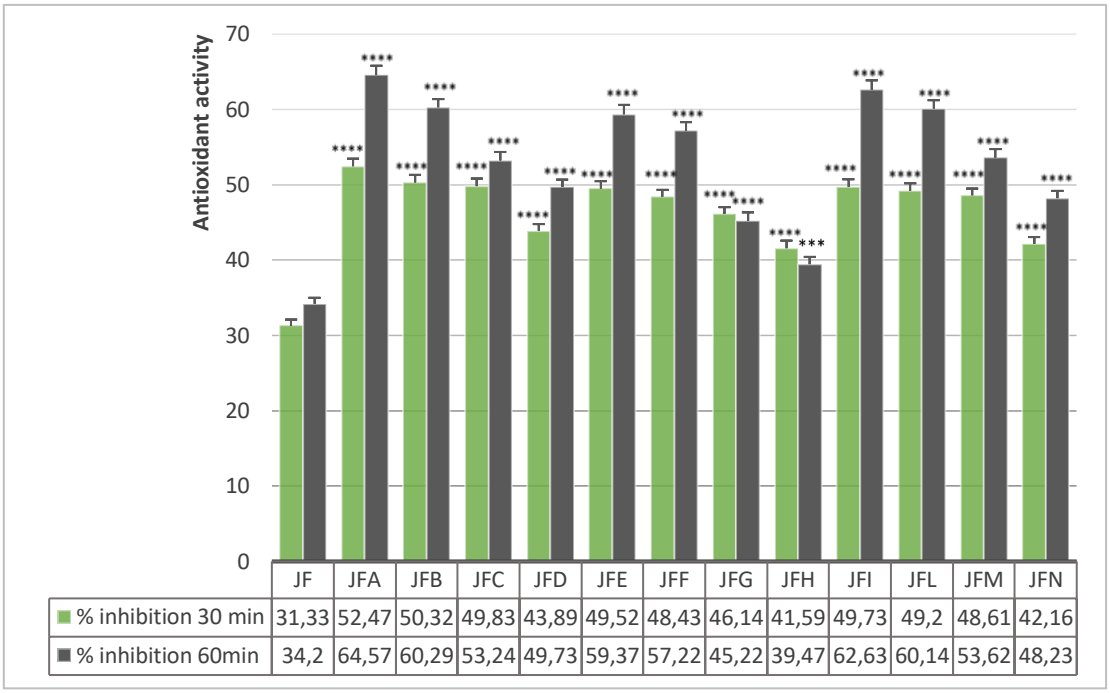
	JF	JFA	JFB	JFC	JFD	JFE	JFF	JFG	JFH	JFI	JFL	JFM	JFN
% Inhibition	96,87	115,46	111,78	110,77	107,64	112,32	110,84	109,77	107,43	118,87	113,92	109,57	97,21

Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative controls (control juice).

Fig. 22. ABTS radical scavenging activity of control and enriched juices

The potential of *C.* × *clementina* juice and enriched juices to inhibit lipid peroxidation was assessed using the β-carotene bleaching test (Figure 23). Generally, enrichment increase the protection on lipid peroxidation. Interesting results were observed for JFA followed by JFI. Sample enriched with dried leaves extract at concentration of 20% w/v showed an increase of 21 and 30% in β-carotene bleaching test, respectively after 30 and 60 minutes of incubation, compared to JF. JFI, enriched with 20% w/v of leaves and peels extracts (1:1), showed an increased protection of lipid peroxidation of 18 and 27%, respectively after 30 and 60 minutes of incubation. Similar result was found with juice enriched with 20% w/v peels extract (JFE), that reach

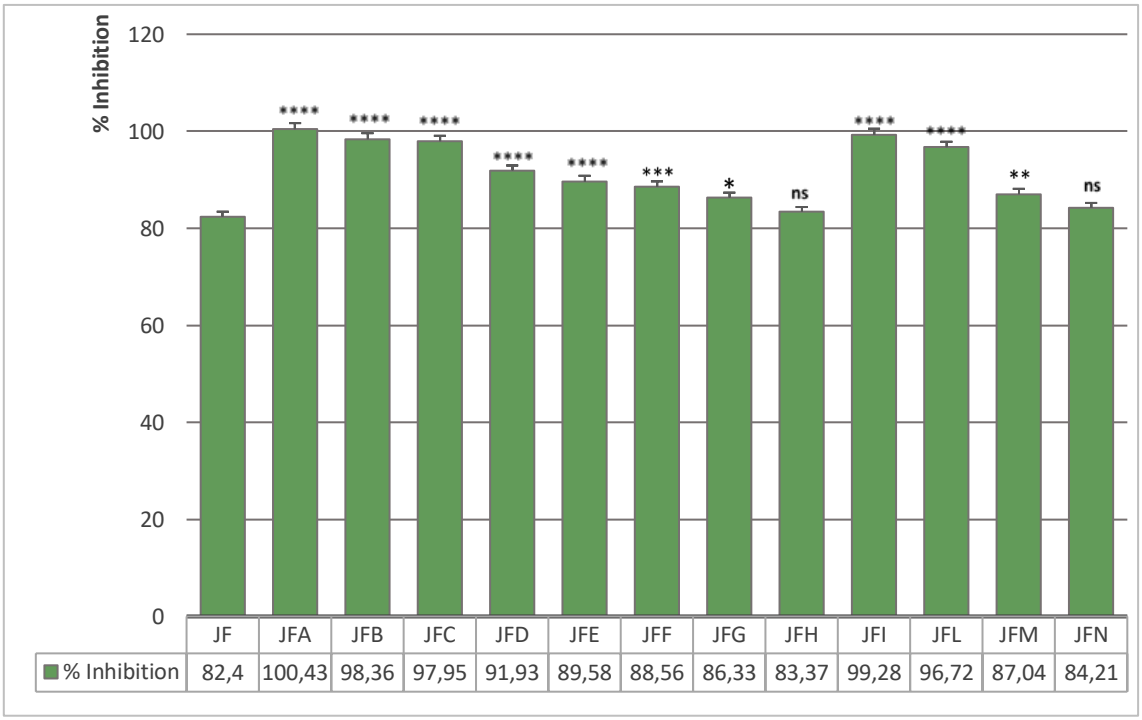
percentage of 18 and 25%, respectively after 30 and 60 minutes of incubation, compared to JF.



	JF	JFA	JFB	JFC	JFD	JFE	JFF	JFG	JFH	JFI	JFL	JFM	JFN
% inhibition 30 min	31,33	52,47	50,32	49,83	43,89	49,52	48,43	46,14	41,59	49,73	49,2	48,61	42,16
% inhibition 60min	34,2	64,57	60,29	53,24	49,73	59,37	57,22	45,22	39,47	62,63	60,14	53,62	48,23

Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative controls (control juice).

Fig. 23. Protection of lipid peroxidation by control and enriched juice



	JF	JFA	JFB	JFC	JFD	JFE	JFF	JFG	JFH	JFI	JFL	JFM	JFN
% Inhibition	82,4	100,43	98,36	97,95	91,93	89,58	88,56	86,33	83,37	99,28	96,72	87,04	84,21

Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, **p< 0.01, *p< 0.1 compared with the negative controls (control juice).

Fig. 24. FRAP inhibitory activity of control and enriched juices

A promising FRAP values were observed in all tested samples (Figure 24). The antioxidant activity in enriched juice decreased as follow: JFA > JFI > JFB, with values of 100.43, 99.28, and 98.36 μg Fe(II)/g, respectively. Among them JFA, determined an increase of 28% in antioxidant activity compared to JF.

Pearson's correlation coefficient was calculated (Table 35). A positive correlation between TPC, TFC, TCC and antioxidant activity was found. In particular, *r* values of 0.88 and 0.97 were found between TPC and DPPH and β-carotene bleaching test after 30 min of incubation. Moreover, TFC was positively correlated with DPPH and FRAP test with *r* values of 0.93 and 0.92, respectively. Regarding TCC a positive correlation was found only with β-carotene bleaching test after 60 min of incubation ($r = 0.93$).

Table 35. *Pearson's* correlation between phenolic compounds content in investigated samples and activity

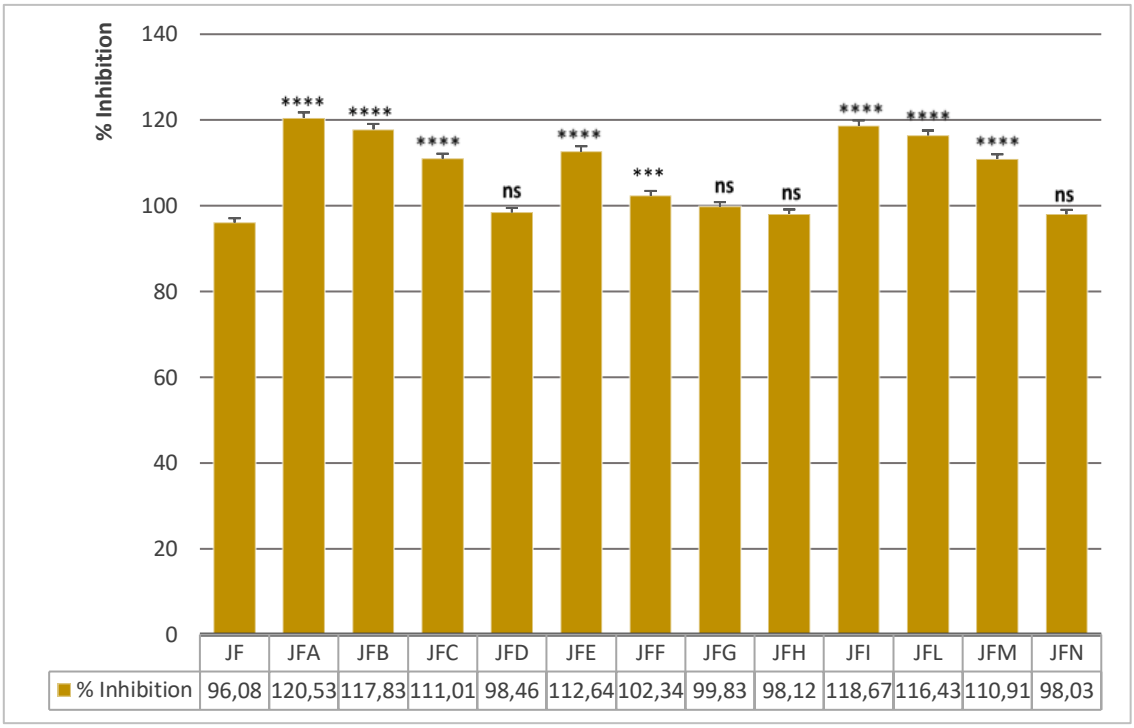
	DPPH test	**ABTS test**	**β-carotene bleaching test t 30 min**	**β-carotene bleaching test t 60 min**	**FRAP test**
TPC	0.83	0.88	0.97	0.87	0.85
TFC	0.93	0.84	0.93	0.86	0.92
TCC	0.66	0.80	0.91	0.93	0.59

4.5.3 Hypoglycaemic and hypolipidemic activity

Following our previously investigation regarding the potential of clementine by-products to inhibit carbohydrate hydrolysing enzymes and lipase, herein we report the results obtained from *in vitro* studies on enriched juice. For this purpose, α-amylase, α-glucosidase and lipase enzymes were selected and used in our investigation.

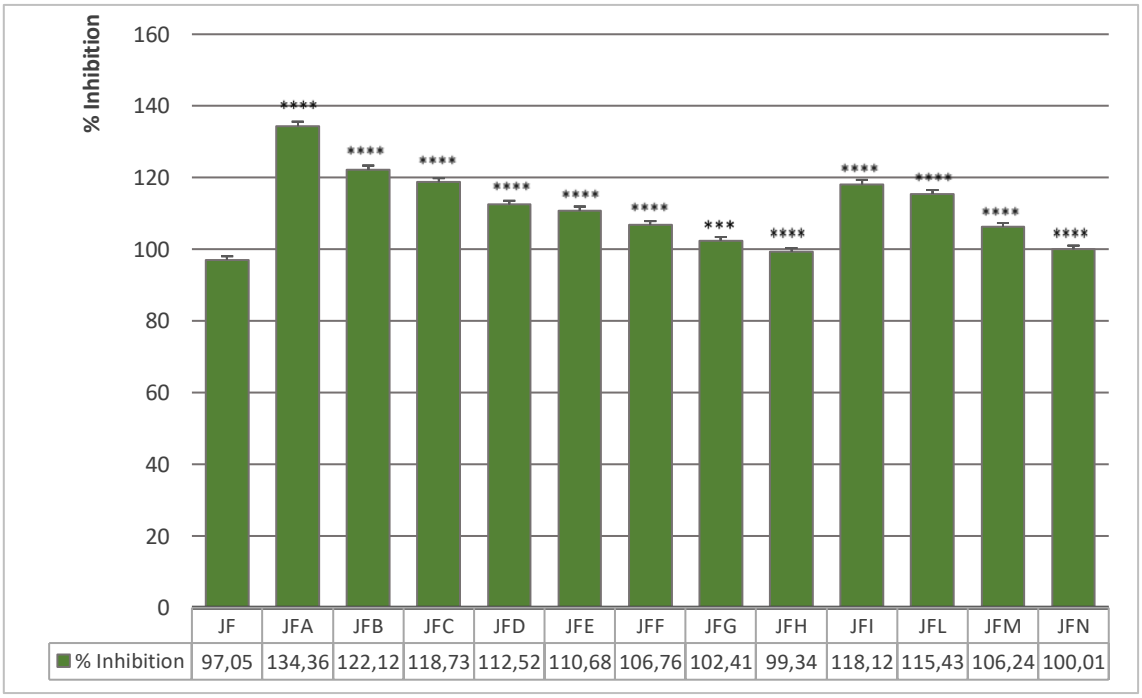
According with figure 25 the juice enriched with leaves extract at concentration of 20% w/v showed an increase of 25% in α-amylase inhibitory activity followed by JFI (22 %) as compared to the juice control. Interesting results were also observed for JFB and JFL (enriched with concentration with 15% w/v of both CO2 and CO2 + BC3 (1:1), respectively) with increase of 22 and 20% in enzyme inhibitory activity. Generally, analysis of data evidenced that enriched juices are more active against α-amylase.

Juice were tested also against α-glucosidase (Figure 26). The hypoglycaemic activity of enriched juice following the rank: JFA > JFB > JFC with values of 134.36, 122.12, and 118.73%, respectively. It is noteworthy that juice enriched with leaves extracts independently by applied concentration are more active than peels extracts enriched juices. With these samples an increase of α-glucosidase inhibitory activity ranging from 22 to 37% was found for JFC and JFA, respectively. Among the others JFI showed an enzyme inhibitory activity about 21% compared to juice control.



Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative controls (control juice).ns: not significant.

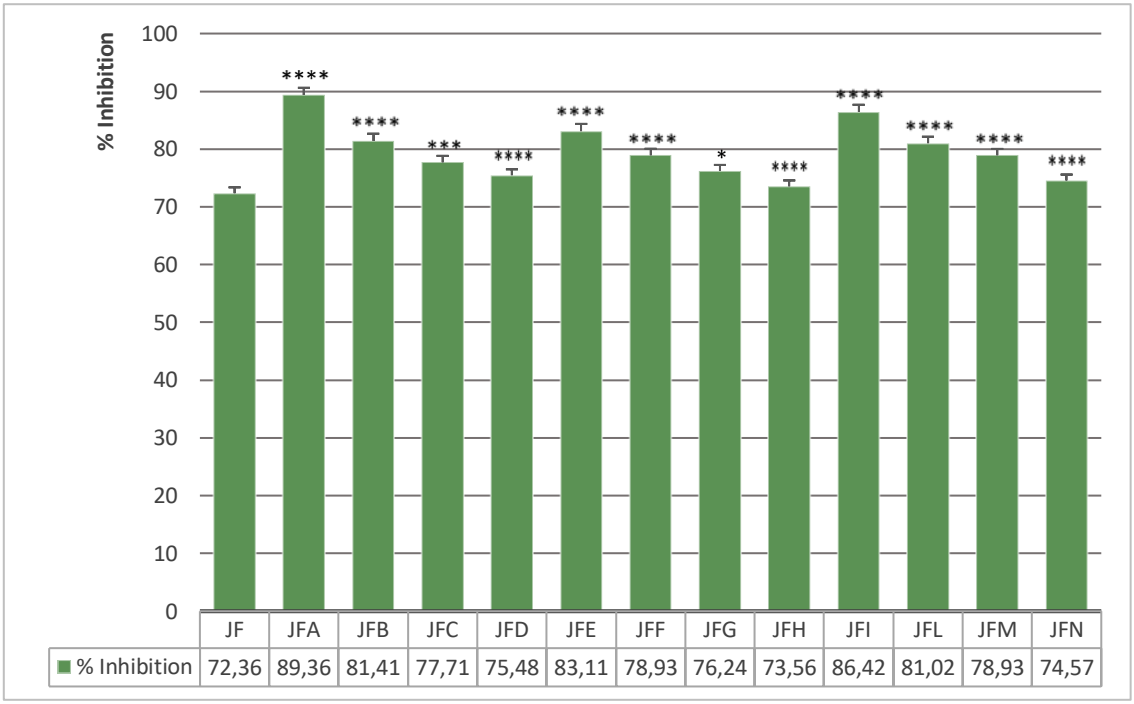
Fig. 25. α-Amylase inhibitory activity of enriched juice and control juice



Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, compared with the negative controls (control juice).

Fig. 26. α-Glucosidase inhibitory activity of enriched juice and control juice

All juice samples were able to inhibit the pancreatic lipase in a concentration-dependent manner as showed in Figure 27. A promising hypolipidemic potential was found with JFA with enzyme inhibition of 89.36% followed by JFI (86.42%). Similar value was observed for JFE. Juice enriched with 20% w/v of leaves extract showed an increase of enzyme inhibitory activity of 17%.



Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, ***p< 0.001, compared with the negative controls (control juice).

Fig. 27. Lipase inhibitory activity of enriched juice and control juice

Pearson's correlation was calculated (Table 36). In particular, the higher positive correlations were found between TFC and α-amylase, α-glucosidase and lipase with r = 0.98 and 0.97, and 0.94 respectively. Positive correlations were also observed between TPC and α-amylase (r = 0.91) and α-glucosidase (r = 0.91). Similarly, TPC and TCC was positively correlated with lipase with r = 0.87, and 0.84 respectively.

Table 35. *Pearson's* correlation between phenolic compounds content in investigated samples and activity

	α-Amylase	**α-Glucosidase**	**Lipase**
TPC	0.91	0.91	0.87
TFC	0.98	0.97	0.94
TCC	0.80	0.73	0.84

4.5.4 Sensory analysis

The sensory assessment is the main aspect for designing new foods because changes in aroma, texture or colour of the original matrices can significantly affect the consumer's acceptance. The evaluation was done using 9-point structured scale, with 9 being the best and 1 the worst product quality. Results of sensory analysis of clementine juice are reported in Table 37.

Colour is the first sensorial quality that attracts attention and influences the consumer's choice. In the regards both colour and appearance of peels enriched juice resulted more attractive than that of the natural juice.

Table 37. Results of sensory analysis of clementine juice

Samples	Appearance	Colour	Odour	Aroma	Sweetness	Acidity	Astringency	Mouthfeel
JF	8.12^{ed}	8.52^{ab}	8.01^{c}	8.14^{ab}	8.23^{a}	7.98^{a}	8.03^{a}	8.26^{a}
JFA	8.01^{f}	8.23^{f}	7.87^{e}	7.96^{e}	8.01^{c}	7.93^{a}	7.86^{de}	7.98^{i}
JFB	8.04^{f}	8.28^{ef}	7.93^{d}	7.98^{de}	8.12^{abc}	7.95^{a}	7.89^{cd}	8.01^{h}
JFC	8.08^{e}	8.33^{def}	7.98^{d}	8.02^{dc}	8.14^{abc}	7.96^{a}	7.91^{cb}	8.15^{cb}
JFD	8.11^{e}	8.49^{ab}	8.00^{c}	8.09^{ab}	8.18^{ab}	7.98^{a}	7.96^{ab}	8.17^{b}
JFE	8.32^{a}	8.55^{a}	8.11^{a}	8.03^{c}	8.03^{bc}	7.95^{a}	7.87^{de}	8.06^{fg}
JFF	8.30^{a}	8.54^{a}	8.07^{b}	8.09^{b}	8.14^{abc}	7.95^{a}	7.92^{cb}	8.10^{cde}
JFG	8.25^{b}	8.53^{b}	8.06^{b}	8.12^{ab}	8.17^{ab}	7.96^{a}	7.98^{b}	8.12^{cd}
JFH	8.22^{b}	8.52^{ab}	8.02^{c}	8.16^{ab}	8.21^{a}	7.97^{a}	8.02^{a}	8.16^{cb}
JFI	8.11^{ed}	8.38^{cde}	8.07^{b}	8.10^{ab}	8.05^{abc}	7.94^{a}	7.85^{e}	8.04^{gh}
JFL	8.13^{cd}	8.42^{bcd}	8.04^{b}	8.12^{ab}	8.13^{abc}	7.96^{a}	7.89^{cd}	8.08^{ef}
JFM	8.14^{c}	8.46^{abcd}	8.05^{b}	8.13^{ab}	8.16^{abc}	7.98^{a}	7.92^{cb}	8.12^{de}
JFN	8.12^{ed}	8.52^{ab}	8.00^{c}	8.15^{a}	8.20^{a}	7.98^{a}	7.98^{b}	8.13^{bcd}
Sign.	**	**	**	**	**	ns	**	**

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. **p< 0.05 Means in the same column with different small letters differ significantly (p< 0.05). Sign: significant.

The same observation could be done for odour, and in fact, JFD obtained the highest score. Regarding aroma, testers found no significant difference between peels enriched juice and the control. The control juice resulted the most acceptable for sweetness, astringency and mouthfeel. The addition of the extracts did not cause a reduction in products acceptance in relation to the attribute of acidity.

4.6 Pasteurization process

Thermal processing is major processing technology in the food industry. The purpose of thermal processing was to extend the shelf life of food products without compromising food safety. Pasteurization is the process that uses relatively mild heat treatment to foods to kill key pathogens, and inactivate vegetative bacteria and enzymes to make food safe for consumption. Pasteurization is based on time-temperature combination processes applied to food products to achieve intended target lethality. We decided to pasteurize the 20% w/v enriched juice samples to evaluate the impact of the process on the phytochemical content and bioactivity of obtained functional products.

4.6.1 C × *clementina* juice enriched pasteurized quality parameters

The *C.* × *clementina* pasteurized enriched juice were investigated for their quality parameters such as TSS, pH, acidity and colour analysis (Table 38).

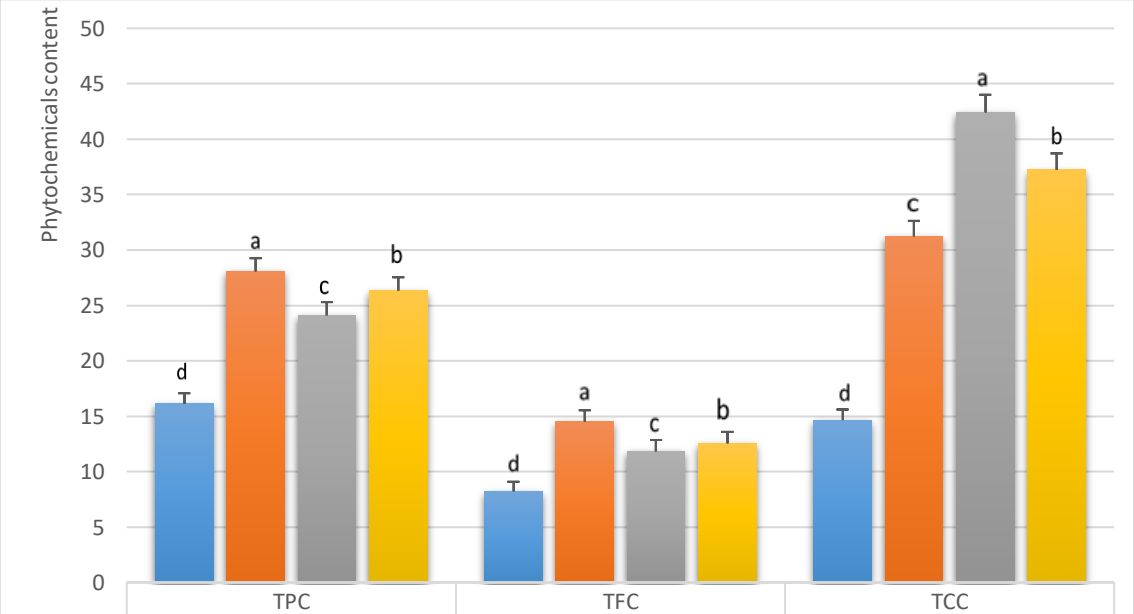
Table 38. Pasteurized juice samples and quality parameters

Samples	°Brix	pH	Acidity (g/100mL)	Croma value (C*)	Hue angle in degrees	Hue Angle in radians
JPF	10.11 ± 1.52^{cb}	3.70 ± 0.53^{a}	0.75 ± 0.07^{a}	30.90 ± 1.91^{a}	75.82 ± 2.21^{d}	1.32 ± 0.09^{a}
JPFA	10.09 ± 1.51^{c}	3.71 ± 0.53^{a}	0.76 ± 0.08^{a}	29.04 ± 1.88^{b}	76.19 ± 2.25^{c}	1.32 ± 0.09^{a}
JPFE	10.13 ± 1.53^{a}	3.69 ± 0.51^{a}	0.75 ± 0.06^{a}	29.51 ± 1.87^{b}	76.96 ± 2.28^{b}	1.34 ± 0.09^{a}
JPFI	10.11 ± 1.52^{ab}	3.7 ± 0.52^{a}	0.74 ± 0.07^{a}	29.16 ± 1.86^{b}	77.05 ± 2.31^{a}	1.34 ± 0.09^{a}
Sign.	**	ns	ns	**	**	ns

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. **p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05). Sign: significant.

The process did not result in significant changes compared to untreated juice. Minimal reduction (5%) were observed in the colorimetric measurement.

The TPC, TFC, and TCC after pasteurization process was evaluated (Figure 28). Results evidenced an increase of phytochemicals content in all samples.



Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. **p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05).

Figure 28. Phytochemicals content in pasteurized juices

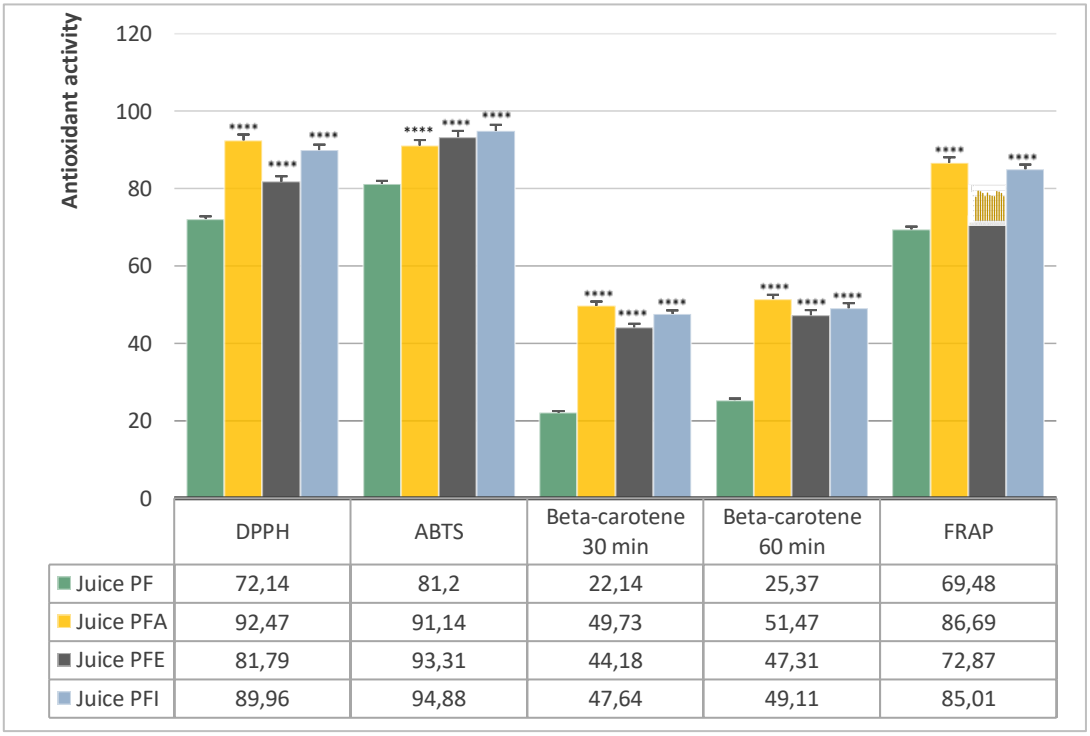
The total phenols content decreased as follow: JPFA > JPFI > JPFE, with values of 28.06, 24.01, and 24.34 mg CAE/100 mL, respectively. Furthermore, the results showed that enrichment with 20% w/v leaves extract had increased in the TPC by 12% compared to JPF. The enrichment with 20% w/v leaves and peels (1:1) extracts determined an increase of 8%. The same value was observed for enrichment with 20% w/v of peels extract.

The TFC in pasteurized enriched juice decreased as follow: JPFA > JPFI > JPFE, with values of 14.51, 12.55, and 11.83 mg QE/100 mL, respectively. The enrichment with 20% w/v of CO2 showed an increase of 6% in the TFC compared with JPF. In JFI and JFE an increase of 4%, was observed.

The TCC in pasteurized enriched juice following the trend JFE > JFI > JFA with values of 42.41, 37.22, and 31.23 mg equivalents of β-carotene/100 mL, respectively. In particular, juice enrichment with 20% w/v of BC3 increase the TCC by 28% compared to JPF. A similar situation was observed with JPFI (22%).

4.6.2 Antioxidant activity

Figure 29 showed the results of antioxidant activity in pasteurized enriched juice. After pasteurization process, a decrease of antioxidant activity of about 20% in DPPH and ABTS test, about 15% in FRAP test and 11.5% in β-carotene bleaching test was observed.



	DPPH	ABTS	Beta-carotene 30 min	Beta-carotene 60 min	FRAP
Juice PF	72,14	81,2	22,14	25,37	69,48
Juice PFA	92,47	91,14	49,73	51,47	86,69
Juice PFE	81,79	93,31	44,18	47,31	72,87
Juice PFI	89,96	94,88	47,64	49,11	85,01

Data are expressed as means ± S.D. (n= 3). DPPH, ABTS and b-carotene bleaching test are express as percentage of inhibition, FRAP test as μM Fe(II)/g. Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative controls (control juice).

Figure 29. Antioxidant activity of *C.* × *clementina* pasteurized enriched juice

In DPPH assay, samples enriched with leaves extract showed a percentage of inhibition of 92.47% with an increase of 20% in antioxidant activity as compared to the control. An increase in DPPH radical scavenging activity about 18% was observed with juice enriched with both leaves and peels extracts (1:1).

The ABTS antioxidant activity following the trend JPFI > JPFE > JPFA, with values inhibition of 94.88, 93.31 and 91.14, respectively. Furthermore, results evidenced that juice enriched with 20% w/v of leaves and peels extract determine an increase of 13% in antioxidant activity as compared to JPF. Similar value was found for JPFE with an increase of 12%.

The potential of *C.* × *clementina* juice to inhibit lipid peroxidation was assessed using the β-carotene bleaching test. Generally, all enriched juice increased the antioxidant activity in comparison to JPF. The greatest activity was observed for juice enriched with leaves extract that increased its activity of 27 and 26% after 30 and 60 minutes of incubation, respectively. Interestingly results were observed for JPFI, enriched with leaves and peels extract (1:1), that increased its activity about 25 and 23% after 30 and 60 minutes of incubation, respectively. Similar results were found, also, for JPFE, enriched with peels extract, that showed an increase of 22 and 21% after 30 and 60 minutes of incubation, respectively.

Samples were tested, also, in FRAP test. FRAP values of 86.69, 85.01, and 72.87 μM Fe(II)/g for JPFA, JPFI, and JPFE, respectively were found. In this assay JPFA, juice enriched with leaves extract determined an increase of 17% in FRAP activity compared to JF. A similar ability was observed, also, with JPFI. The enrichment with peels extract did not cause a significant increase in FRAP.

Pearson's correlation coefficient evidenced a positive correlation between TPC and DPPH and β-carotene bleaching test after 30 and 60 minutes of incubation with *r* values of 0.97, 0.99 and 0.98, respectively (Table 40). Similar positive correlations were found between TPC, TFC and FRAP test with *r* values of 0.87 and 0.88, respectively. TCC positively correlated with ABTS and β-carotene bleaching test at 30 and 60 minutes of incubation with *r* values of 0.95, 0.84 and 0.86, respectively.

Table 40. *Pearson*'s correlation between phenolic compounds in investigated samples and activity

	DPPH test	**ABTS test**	**β-carotene bleaching test t 30 min**	**β-carotene bleaching test t 60 min**	**FRAP test**
TPC	0.97	0.88	0.99	0.98	0.87
TFC	0.96	0.79	0.96	0.95	0.88
TCC	0.63	0.95	0.84	0.86	0.40

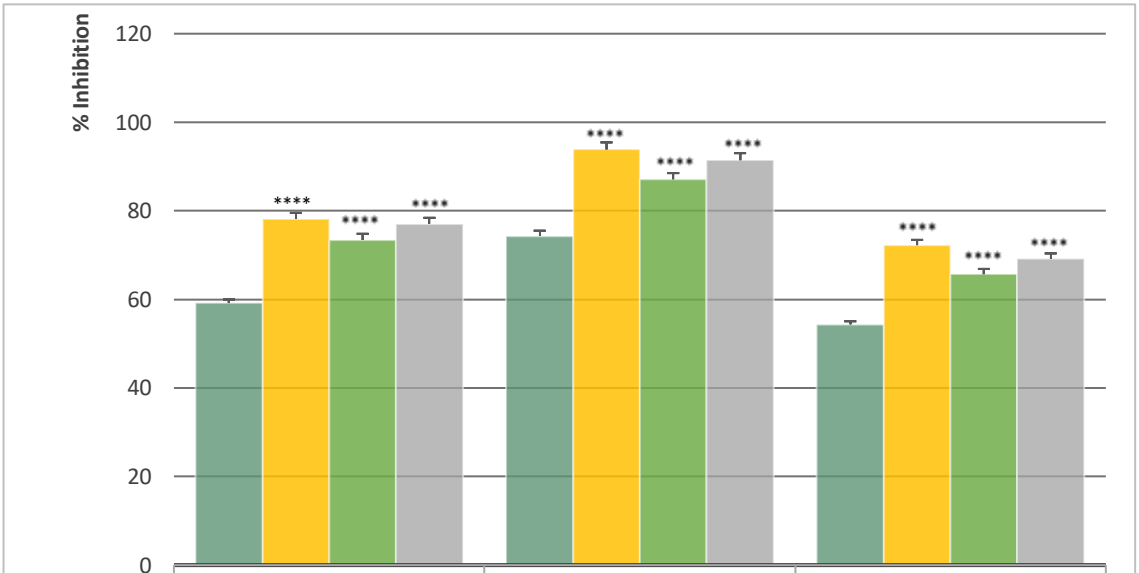
4.6.3 Hypoglycaemic and hypolipidemic activity

The study of enzymes inhibitory activity of pasteurized juices was performed (Figure 30). The pasteurization process caused a decrease in enzyme inhibitory potential about

24 and 20%, respectively for α-amylase and α-glucosidase assay, and about 17% for lipase enzyme.

According with figure 30, in α-glucosidase assay, the pasteurized juice enriched with leaves extract induced an increase of 20% in hypoglycaemic activity followed by JPFI (13%) as compared to the control juice. Moreover, all pasteurized samples were able to inhibit the α-amylase enzyme in a concentration-dependent manner. The pasteurized juice enriched with leaves extract induced an increase of 19% in hypoglycaemic activity followed by JPFI (17%) as compared to the control juice.

A promising hypolipidemic potential was found for JPFA with inhibition of 72.25% followed by JFI with inhibition of 69.19%. A similar value was observed for JPFE. The pasteurized juice enriched with leaves extract determined an increase of 18% of the enzyme inhibitory activity.



	Alpha-amylase	Alpha-glucosidase	Lipase
Juice PF	59,18	74,32	54,36
Juice PFA	78,15	93,85	72,25
Juice PFE	73,42	87,12	65,69
Juice PFI	77,03	91,42	69,19

Data are expressed as means ± S.D. (n= 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α= 0.05): ****p< 0.0001, compared with the negative controls (control juice).

Figure 30. The inhibition of carbohydrate hydrolysing enzymes and lipase inhibitor activity of *C.* × *clementina* pasteurized enriched juice

A positive *Pearson's* correlation coefficient was found between TPC and all conducted tests (r = 1.00) (Table 41).

Table 41. *Pearson's* correlation between phenolic compounds in investigated samples and carbohydrate hydrolysing enzymes and lipase

	α-Amylase	**α-Glucosidase**	**Lipase**
TPC	1.00	1.00	1.00
TFC	0.96	0.98	0.97
TCC	0.82	0.76	0.80

4.5.4 Sensory analyses

The sensory analyses were also carried out for enriched juice samples after pasteurization process. The evaluation was done using 9-point structured scale, with 9 being the best and 1 the worst product quality. Results of sensory analysis of clementine juice are reported in Table 42.

Table 42. Results of sensory analysis of clementine pasteurized enriched juice

Samples	**Appearance**	**Colour**	**Odour**	**Aroma**	**Sweetness**	**Acidity**	**Astringency**	**Mouthfeel**
JF	7.59^{c}	7.94^{b}	7.87^{b}	8.10^{a}	8.01^{a}	7.76^{a}	7.93^{a}	7.89^{a}
JFA	7.47^{c}	7.93^{b}	7.81^{b}	7.81^{c}	7.87^{c}	7.71^{c}	7.84^{b}	7.73^{c}
JFE	8.11^{a}	8.25^{a}	8.07^{a}	7.86^{c}	7.98^{b}	7.73^{b}	7.86^{b}	7.86^{b}
JFI	7.61^{b}	7.96^{b}	7.86^{b}	7.91^{b}	8.00^{a}	7.74^{b}	7.85^{b}	7.85^{b}
Sign.	**	**	**	**	**	**	**	**

Data represent means ± SD (standard deviation) (n = 3). Differences were evaluated by one-way analysis of variance (ANOVA) test completed with a multicomparison Tukey's test. **p< 0.05. Means in the same column with different small letters differ significantly (p< 0.05).

On the basis of the average ratings given by testers, the enriched juice untreated were more accepted compared to pasteurized juice. The pasteurized peels enriched juice was again obtained the best scores for appearance, colour and odour. While, for other parameters the natural juice was resulted more attractive than that of enriched juice.

Overall, the *C.* × *clementina* pasteurized enriched juice was well accepted by the testers, with average scores above 7-8 for all concentration.

4.6.5 PCA enriched juices

For the evaluation of the results (TFC, TPC, TCC, DPPH, ABTS, β-carotene-bleaching test after 30 and 60 minutes of incubation, FRAP, α-amylase, α-glucosidase and lipase) deriving from fresh, pasteurized and enriched juices, the multivariate statistical methods (PCA) was applied.

The projections of the observations on the first two principal component axes were showed in Figure 31. The accessions were distributed on the factor plane. These two coordinates represented 98 % of the total variance (PC1 explained 58 % of total variation, while PC2 explained 36 % of total variation). The first component (PC1) was positively and strongly correlated with TFC, TPC, TCC, DPPH, ABTS, FRAP, α-amylase and α-glucosidase. The second component (PC2) was positively correlated with FRAP, β-carotene bleaching 30 and 60 min incubation, and lipase.

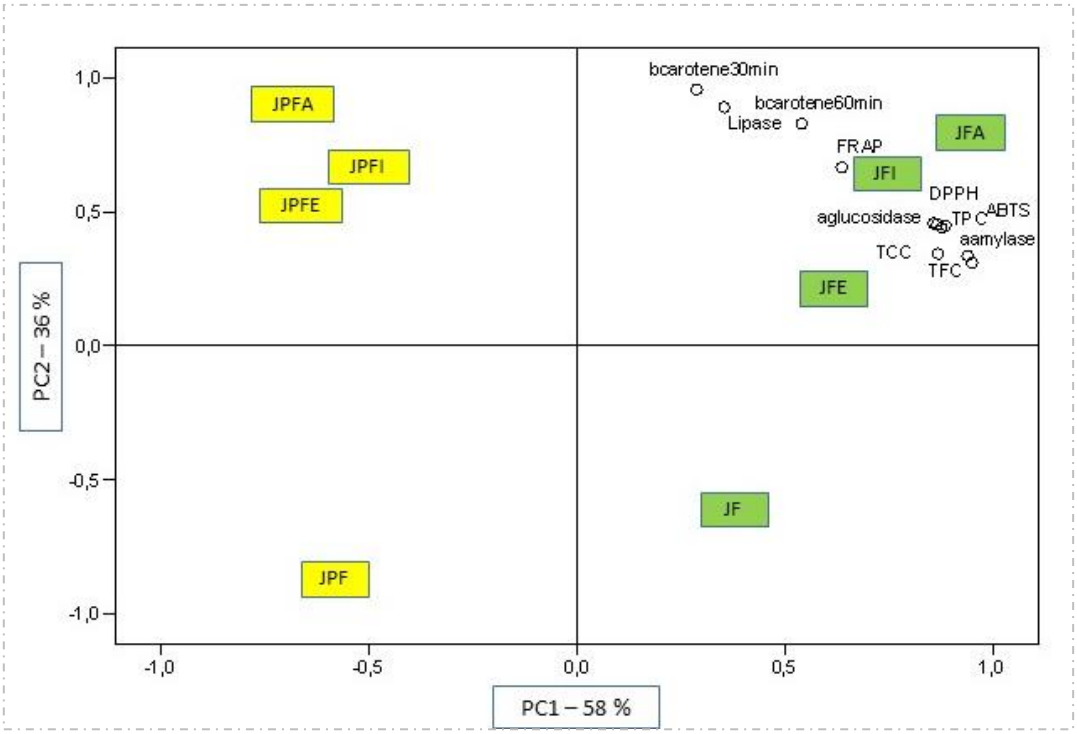


Fig. 31. Principal component analysis plot based on bioactivity attributes of fresh and pasteurized juice samples and enriched juice samples

The scores plot was used to gain an overview of the similarities or differences among the juices (fresh juice JF, enriched fresh juice JFE, JFI, JFA, pasteurized juice JPF and enriched pasteurized juice JPFE, JPFI, JPFA).

Figure 31 also showed the space of juice samples and the bioactive attributes associated with the juice samples, fresh and pasteurized. The spatial distributions of points relating to the juice samples form separate clusters. Fresh juices and enriched fresh juices (JF, JFA, JFI and JFE) were perceived as having better quality characteristics. The group of pasteurized juices (JPF, JPFA, JPFI and JPFE) were characterized by a lower intensity of characteristics describing in the first group.

Antioxidant activity represented a common feature for JFE, JFI and JFA. By PCA model it can be observed showing that enriched fresh juices were characterized by higher TFC, TPC and TCC content as well as by higher DPPH, ABTS, FRAP, α-amylase, α-glucosidase, β-carotene bleaching 30 and 60 min incubation, and lipase activity in comparison to the pasteurized juices. Having in mind the above dependencies, the first principal component (PC1), may be interpreted as the measure of positive characteristics of fresh juices and enriched fresh juices.

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Conclusion

The implementation of circular economy and eco-friendly initiatives creates an industrial system, which allows organisations to recycle the materials to enhance the overall sustainability. This can contribute to innovation and growth in the food and beverage industry. The biomolecules recovered from the by-products can be used to produce functional foods and consequently offer a new opportunity for by-products reutilization. Therefore, food and nutraceutical industries have a common interest to obtain new natural bioactive components to be used as functional food ingredients or nutraceuticals. Functional food products have received enormous attention in the food market due to the growing interest of consumers in "healthy" foods.

Citrus is the most produced tree fruit crop in the world. Almost 33% of the Citrus fruits are industrially processed for juice production, however, a great amount of *Citrus* wastes including peels, segment membrane and seeds are produced.

In this context, the present research project aimed to investigate a model for the reuse of *C.* × *clementin*a Hort. ("Clementine di Calabria, PGI) by-products, in order to propose their utilization for the development of functional beverage useful in prevention and treatment of prediabetic condition, hyperglycaemia and hyperlipidaemia.

For this purpose, the chemical composition and bioactivity of *C.* × *clementina* juice and by-products (leaves and peels) were investigated. The possibility to enrich juice with its by-products in view on a possible reuse of this renewable source of bioactive compounds in terms of circular economy was, also, performed.

Fruit quality parameters, juice nutritional parameters, chemical profile, antioxidant properties, carbohydrate hydrolysing enzymes inhibitory activity as well as lipase inhibitory activity of *C.* × *clementina* Hort. juice, peels and leaves extracts and essential oils were assessed. The influence of area of collection on chemical composition and bioactivity was also analysed.

Generally, the areas of collection positively influenced the quality and bioactivity of the juice. In particular, juice obtained from fruits collected in Corigliano Calabro (JF), the main area in which clementine of Calabria are produced, was characterized by a higher content of bioactive compounds with consequent higher bioactivity as confirmed by PCA.

For this reason, JF was chosen as a matrix to be enriched with *C.* × *clementina* leaves and peels extracts for the development of functional drink.

C. × *clementina* chemical profile revealed that hesperidin, tangeretin, sinensetin, and isoquercetin were the most abundant identified constituents especially in leaves extracts. Among applied extraction techniques, ultrasound-assisted maceration resulted the most promising procedure to obtain extracts characterized by the highest of bioactivity in terms of antioxidant and carbohydrate hydrolysing enzyme inhibition.

For this reason, CO2 sample obtained by ultrasound-assisted maceration in $EtOH/H_2O$ of leaves collected in Corigliano Calabro, was chosen as phytocomplex to be added to JF for the development of the functional beverage.

Hesperidin, sinensetin, tangeretin, and luteolin were the most abundant compounds in *C.× clementina* peels extracts. In particular, the extract obtained by ultrasound-assisted maceration in EtOH from peels collected in Cetraro (BC3), was chosen as phytocomplex to be added to JF, as confirmed by PCA.

Considering that the content of coumarins is strictly regulated in foods, the absence of these phytochemicals in our bioactive samples represents an additional value for their potential industrial application.

Based on obtained data, the enrichment with leaves extracts given the best results and a better impact both on the content of bioactive compounds and bioactivity. In particular, the addition of CO2 at concentration of 20% w/v determined an increase in antioxidant activity of 28, 21, 30, and 28% in DPPH, β-carotene bleaching test (respectively after 30 and 60 min of incubation), and FRAP assay, respectively.

An increase of 22% of ABTS radicals scavenging activity was observed with juice enriched with 20% leaves and peels extracts (JFI, 1:1) sample. Additionally, JFA is more active against α-glucosidase (+37%), α-amylase (+25%) and lipase inhibitory activity (+17%) as compared to JF. Also, after pasteurization process, enrichment with leaves by-products (JPFA) resulted the most promising in the increase of total phytochemicals content and bioactivity compared to the pasteurized control juice. (JPF). This juice showed the highest antioxidant activity in DPPH (+20%), β-carotene bleaching test (+ 26 and +27%, respectively after 30 and 60 min of incubation) and FRAP assay (+ 17%).
A different behaviour was observed in ABTS test in which JPFI resulted the most active (+13%). No significant differences were observed for JPFA and JPFI in enzyme inhibition.

It is necessary to consider that enriched juice is a phytocomplex and its efficacy is due to the combine effect of all the compounds. Indeed, its effect is the results of a synergism of all constituents or reflect only those of the main molecules present at the highest levels. However, it is possible that the activity of the main constituents is modulated by minor abundant compounds. This is due to the ability of these molecules to penetrate cells, to create a lipophilic or hydrophilic linkage and fixation on cell wall. Based on these considerations it is more important the study of the phytocomplex rather than its constituents because the concept of synergism appears to be more meaningful also when *in vivo* studies will be performed.

In conclusion, the promising bioactivity of enriched juices and comforted by sensory analysis data we can propose *C. × clementina* by-products as a promising source of bioactive compounds useful for formulation of functional drink or foods for preventing diseases associated to oxidative stress with particular reference to hyperglycaemia and hyperlipidaemia. Moreover, the high antioxidant potential of these extract allows us to propose their use as food additives able to extent the *shelf-life* of different foods products.

The present results encourage researchers to continue their working with further *in vivo* studies in order to assess the impact of functional juice on pathological models. Additionally, further investigations will be conducted to evaluate the bioaccessibility, bioavailability as well as possibility of hypovitaminoses due to fat reduction of *C. × clementina* juice and their derived products.