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"Isolation, characterization and biological evaluation of onconutraceuticals compounds in the Mediterranean area: new tools for the treatment of oncological diseases"

Scientific Disciplinary Sector

CHIM/08 – Medicinal Chemistry

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*proteins involved in the UPR (c-Caspase-12 and c-ATF6) were also evaluated. α-Tubulin was used to check equal loading of protein extracts. The relative fold change vs untreated cells, set as 1, is shown in the graph. (**B**) Densitometric analysis of western blotting. (**C**) Measurement of intracellular ROS detected with DCFH-DA. H2O2 (800 μM, 4h) was used as positive control. (**D**) s-XBP1 and ATF4 mRNA ER stress hallmarks were assessed with q-PCR. GAPDH was used as housekeeping control. The 2–ΔΔCT method was employed to calculate the relative quantities of mRNA. Results are expressed as fold change relative to untreated cells. Data are showed as the mean ± SD of three different experiments performed in triplicate. * $p < 0.05$ vs. Ctrl; ** $p < 0.01$ vs. Ctrl; *** $p < 0.001$ vs. Ctrl.*

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ABSTRACT

The main objective of the PhD project was the evaluation of the onconutraceutical potential of food matrices from the Mediterranean area, in particular *Citrus sinensis* (sweet orange), *Humulus lupulus* (hops) and *Lycium barbarum* (goji berries). It is known that the daily intake of fruit and vegetables, the introduction of foods rich in bioactive substances into the diet and a healthy and correct lifestyle could represent possible strategies aimed at reducing the incidence of cancer. The discipline that deals with the study of foods with anticancer activity is Onconutraceutics. In detail, the bioactive extracts of the plant matrices under study were characterized by high-performance liquid chromatography experiments coupled with high-resolution mass spectrometry studies. The results obtained highlighted the presence of a wide variety of bioactive molecules, including Nobiletin in *C. sinensis*, Xanthohumol in *H. lupulus* and Rutin in *L. barbarum*, whose antitumor properties are described in the literature.

Initially, the methanolic extracts of *C. sinensis* and *H. lupulus* were fractionated by semipreparative reverse-phase liquid chromatography, yielding 4 and 3 fractions, respectively. Among these we obtained two main bioactive aliquots: NCF from *C. sinensis*, containing polymethoxyflavones and in particular Nobiletin and XCF from *H. lupulus*, consisting of prenylflavonoids whose main compound was represented by Xanthohumol. The purpose of the fractionation was to obtain fractions with a high onconutraceutical potential.

In order to evaluate the potential use of NCF, XCF or their combination as chemo-sensitizing agents and to reduce the cytotoxicity of chemotherapy treatments, we investigated the potential synergistic effects of these natural compounds with 5-

Fluorouracil and Oxaliplatin (FOX) on CR-CSphC isolated from both naïve primary CRC and liver metastases of chemotherapy-refractory patients.

The results obtained demonstrated that the administration of NCF, XCF and their combination (Mix) significantly reduced the proliferation of CR-CSphC, and consequently the expansion of metastatic CR-CSC in chemotherapy-refractory patients. Interestingly, these natural plant-derived compounds, singly or in combination, are useful as additive molecules to chemotherapy, possibly limiting antitumor cytotoxicity against normal cells.

The results obtained, therefore, could provide a starting point for the use of these matrices as adjuvants of the classic pharmacological treatment and prevention of colorectal cancer.

The next phase of the research project concerned the evaluation of the potential antioxidant and anticancer properties of the phytocomplexes extracted from five Mediterranean area matrices on tumor and healthy cells. Analysis of the initial screening data showed that *L. barbarum* extract (LBE) had the highest antiproliferative capacity on the HeLa cervical cancer cell line and had no significant effect on MCF10A healthy cells. Subsequently, since the IC_{50} on HeLa cells was slightly elevated and since MCF10A cells were used as healthy cells, it was thought to use the corresponding breast cancer cell line MCF-7 to evaluate the possible biological properties of the LBE extract.

The obtained results showed that goji berry extract is able to reduce the viability of MCF-7 tumor cells without having a significant effect on MCF10A healthy cells. An interesting aspect that emerged is also a double behaviour on reactive oxygen species, as it has both an antioxidant and a pro-oxidant action. The antiproliferative activity of LBE is probably due to the pro-oxidant action exerted inside the tumor cells in the first hours of administration. The state of imbalance within the cell has led to disabling the structures

necessary for cell survival and proliferation and inducing cell death, through a strong activation of endoplasmic reticulum stress.

In conclusion, the obtained results further confirm the belief in the health benefits deriving from *Lycium barbarum*, as a very promising natural medicine in the prevention and treatment of cancer due to their high content of active compounds. The anticancer actions of LBE together with the lack of toxic effects on normal cells make the constituents of *L. barbarum* attractive chemopreventive agents.

CHAPTER I:

Isolation, characterization and biological evaluation of onconutraceuticals compounds in the Mediterranean area: new tools for the treatment of oncological diseases

1. Introduction

The main purpose of nutrition is to provide, through food intake, energy and components (water, proteins, carbohydrates, fats, vitamins and minerals) essential for the fulfillment of the vital functions and nutritional needs of each individual. Since ancient times it has been known that lifestyle and diet have significantly effect on the human health and wellbeing, and also play a crucial role in the prevention of diseases. On the other hand, incorrect dietary habits associated with a sedentary lifestyle have proved to be contributory causes of various pathologies, from cardiovascular to cancer (Santini et al., 2017). The discipline that studies "the food components or the active ingredients present in foods that have positive effects for well-being and health, including the prevention and treatment of diseases" is the "Nutraceutical". This term was coined in 1989 and derives from the union of the terms "Nutrition" and "Pharmaceutical" (DeFelice, 1995). It identifies a food or part of a food, which can be of vegetal or animal origin, that has a beneficial pharmaceutical activity beyond its nutritional value. They are often confused with food supplements, which do not have a specific pharmacological effect on health condition. Food supplements are often confused with nutraceuticals, but, while nutraceuticals must have a proven clinical efficacy beyond their nutritional value, the food supplements do not necessarily have a specific proven action on a health condition. Supplements can compensate and/or have a beneficial effect due to the addition of specific components, in the face of a lack of a micro or macro nutrient in the body. They

do not have necessarily any proven pharmacological effect. Nutraceuticals, on the opposite, are formed by many active substances extracted from vegetal or from animal origin food, which are concentrated and administered in the suitable pharmaceutical form, and must have a pharmacological effect in addition to their nutritional value. The nutraceuticals use in the daily diet has the utility of preventing the onset of pathological conditions by avoiding or reducing the use of drug therapy, therefore it is "beyond the diet, before the drug" (Santini, 2014). Plants produce numerous organic compounds that do not seem to have a direct function on their own growth and development, and these substances are known as secondary metabolites, secondary compounds or natural products. Unlike primary metabolites, such as chlorophyll, amino acids, nucleotides or simple carbohydrates, secondary products have no generally recognized roles in the processes of photosynthesis, respiration, solute transport, translocation, nutrient assimilation and differentiation. The study of plant secondary metabolites has many practical applications. Due to their biological activity against herbivorous animals and microbes, many of these substances are used as insecticides, fungicides and pharmaceutical products, while others are used as perfumes, flavors, medicinal drugs and raw materials for industry. In many Eastern countries, traditional herbal medicines and their preparations have been used for thousands of years to maintain good health and treat diseases. In recent years they have attracted the attention of many researchers, for their pharmacological efficacy combined with reduced side effects. It is known that the therapeutic effect of a plant extract is based on the synergistic effect of its constituents, that is, the set of all the substances present responsible for the specific therapeutic action of the plant, defined as a phytocomplex. In addition to the active ingredient, the phytocomplex also contains inert substances and substances that regulate pharmacological activity. Compared to the single active ingredient, the phytocomplex has

a much more complex composition, and this can be considered the main reason why quality control on plant extracts is more difficult than that applied to Western medicine. The deepening of the properties of natural compounds at the molecular and cellular level, could allow to identify the pharmacological targets of the phytocomplexes of interest, to evaluate their global activity and open a way for the identification of the most significant biomarkers in the modulation of the inflammatory process.

Nowadays, the challenge is more and more focusing on prevention more than on diseases cure and therapy. For these reasons there is a growing interest in alternative and more "natural" approaches to preventive actions with respect to pharmacologic therapy (Das et al., 2012, Rautiainen et al., 2016). For this reason, nutraceuticals can be considered important elements in "proactive medicine". An approach that consists in preventing the onset of a disease rather than pharmacologically treating the symptoms that are now evident. Figure 1 summarizes the overall picture of the nutraceutical approach to health issues and prevention.

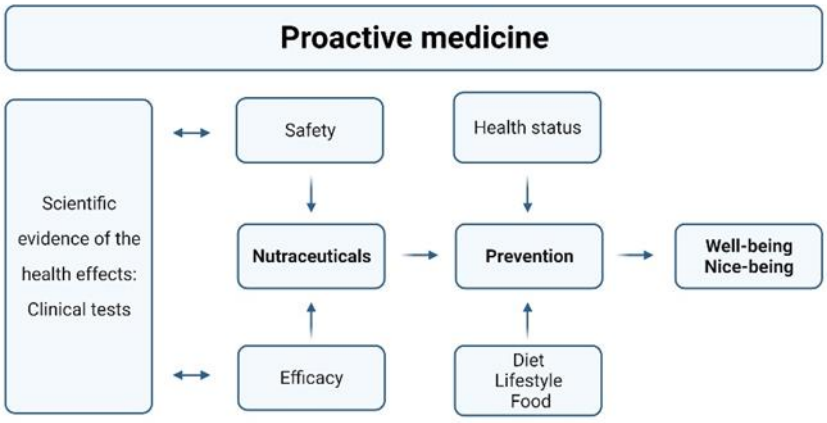


Figure 1. *Proactive medicine nutraceutical approach.*

1.1. Onconutraceutical

The incidence of tumors, an increasingly predominant event on the global scene, has prompted scientific research to focus its efforts on the chemical identification of phytocomplexes with chemopreventive activity and on understanding the mechanisms of action.

Between 30 to 40% of cases are reportedly preventable through diet modification, adequate body weight management, and physical activity (Manson, 2003). Recently, nutraceuticals have gained much attention in cancer research because of their pleiotropic effects and relatively non-toxic behavior (Nair et al., 2010).

Almost all cells in the body can undergo neoplastic transformation, i.e., the transformation of a healthy cell into a tumor cell following a DNA lesion caused by a random or hereditary error or by a mutagenic agent. If the lesion is not repaired, the altered DNA sequence can cause genes to turn on or off that interfere with the normal functioning of the cell. Neoplastic transformation occurs when enough mutations accumulate to change a cell's ability to survive and multiply.

It can be argued that cancer is due to a complex multi-step process in which cumulative genetic and epigenetic changes occur in a normal cell. The causes of the development of cancer are multiple and complex and probably depend on two or three etiological factors which act in sequence or simultaneously. The term carcinogen identifies a chemical, physical or biological agent (molecule or chemical mixture, radiation, viral, bacterial, fungal, animal, condition of exposure) capable of causing tumors or favoring their onset and propagation.

Cancer development is characterized by three stages: initiation, promotion, and progression (Figure 2).

- *Initiation*: The first stage in tumor development is initiation, a rapid and irreversible process during which an alteration (mutation) of the cell genetic material stimulates the cell to become cancerous. Genotoxic DNA damage can be spontaneous or caused by an exogenous or endogenous carcinogen which involves the onco-genes activation or tumor suppressor genes inactivation.
- *Promotion*: is a process characterized by the transformation of a preneoplastic cell initiated by agents that stimulate its proliferation, such as growth factors, hormones and UV radiation. This leads to the transmission of the transformed genotype to daughter cells and the accumulation of further mutations.
- *Progression*: The final stage of neoplastic transformation, progression, involves uncontrolled cell growth, increased invasiveness and metastatic potential, and the new blood vessels formation (angiogenesis) (Ramos, 2008).

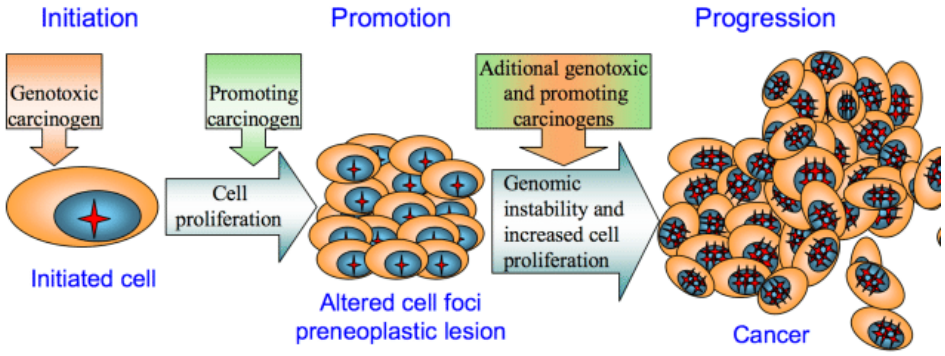


Figure 2. *Developmental stages of carcinogenesis.*

Essential characteristics ("hallmarks") have been identified that tumor cells have in common and that differentiate them from normal cells:

- A normal cell can divide only a limited number of times, a tumor cell instead is capable of multiplying without stopping (*replicative immortality*).

- A normal cell divides only when needed, a tumor cell instead proliferates even in the absence of stimuli (*uncontrolled proliferation*).
- A normal cell responds to signals that block proliferation, while a tumor cell ignores them (*insensitivity to factors that block cell growth*).
- A damaged or no longer needed normal cell responds to signals that cause it to die (programmed cell death is called apoptosis), a cancer cell instead becomes immortal (*resistance to apoptosis*).
- A tumor cell acquires the ability to migrate from the tissue of origin and give rise to secondary tumors in other tissues or organs (*invasiveness and metastasis*).
- A tumor cell acquires the ability to stimulate the formation of new blood vessels, which are needed to nourish the tumor (*promotion of angiogenesis*).
- A cancer cell uses different mechanisms than a normal cell to produce the energy it needs (*reprogramming of energy metabolism*).
- A tumor cell easily acquires new DNA alterations, which can affect more or less extensive areas of the chromosomes (*genomic instability*).
- A cancer cell evades the anti-cancer actions of the immune system (*evasion of immune responses*).
- A tumor cell interacts with an inflammatory microenvironment: Chronic inflammation can promote the onset and growth of the tumor, which then contributes to maintaining this inflammatory state (*cancer-associated inflammation*).

A human tumor represents the final stage of a long polyphasic process that takes place in the phenotype and in the cellular genotype. The main cause therefore consists in

alterations of the genome at the level of expression or function of the genes responsible for the control of cell growth and differentiation (Figure 3).

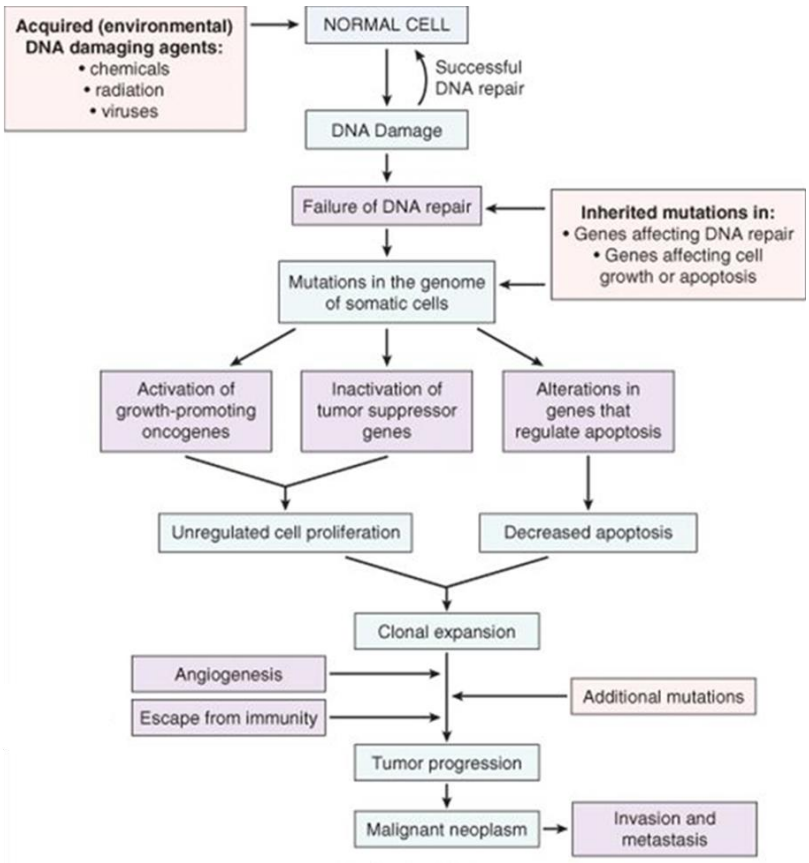


Figure 3. *Schematic of the molecular basis of cancer.*

The genetic alterations responsible for tumors are localized at the level of oncogenes and tumor suppressor genes. In general, these are somatic events even if germline mutations can determine a (hereditary) predisposition to the disease. A single genetic alteration is (generally) not sufficient to determine the disease, which develops with a chronic multiphase process during which the tumor cells acquire numerous genetic alterations.

Oncogenes are mutated forms of genes that positively control cell growth and differentiation (proto-oncogenes). When a proto-oncogene mutates into an oncogene (activation), it works even when under normal conditions it shouldn't.

Tumor suppressor genes are normal genes whose function is implicated in the negative control of proliferation, maintenance of genomic stability, and apoptosis. When a tumor suppressor gene malfunctions (inactivation), the cell can grow out of control, fail to repair DNA properly, or fail to respond to signals for apoptosis. In general, the activation of oncogenes is due to mutations acquired during life, while the inactivation of tumor suppressor genes can be either acquired or inherited.

In the field of Nutraceutical studies, particular attention is paid to the Onconutraceuticals area, identified as the study of nutraceutical substances with chemopreventive and antitumor activity, but also the study of the preparation methods and routes of administration of the compounds used to favor them bioavailability. It is based on epidemiological studies, preclinical studies, on *in vitro* and *in vivo* models and therefore on cell cultures and animal models.

The use of nutraceuticals in cancer therapy is mainly aimed at:

- the chemoprevention of tumors (primary prevention);
- the identification of compounds to be associated with anticancer therapy for an additive or synergistic action (adjuvant therapy), in order to decrease drug concentrations and, consequently, the side effects associated with current anticancer treatments;
- the reduction of the phenomenon of therapy resistance (chemo, hormone and radiotherapy).

Chemo preventive compounds must be safe, inexpensive, accessible and with clear and defined molecular mechanisms. Nutraceuticals useful for health include essential com-pounds such as selenium, zinc, calcium and vitamins C, D, E and B and non-essential compounds such as carotenoids, polyphenols, conjugated acid and fatty acids. They are obtained from different sources such as plants, fruits and animals (Arora and Jaglan,

2016). Nutraceuticals act in cancer prevention and treatment exerting their action on multiple signaling pathways, inhibiting cancer cells proliferation and differentiation, inhibiting efflux transporters such as P-gp or reducing chemotherapy drugs toxicity such as cardiotoxicity and hepatotoxicity. They also act on cell cycle control, regulate apoptosis, inflammation, angiogenesis and metastasis processes, that could be targets for anticancer therapies. Furthermore, natural compounds can also have antioxidant activity, through protection from the action of free radicals that alter DNA and cell membrane's structure (Figure 4) (Lecour and T Lamont, 2011).

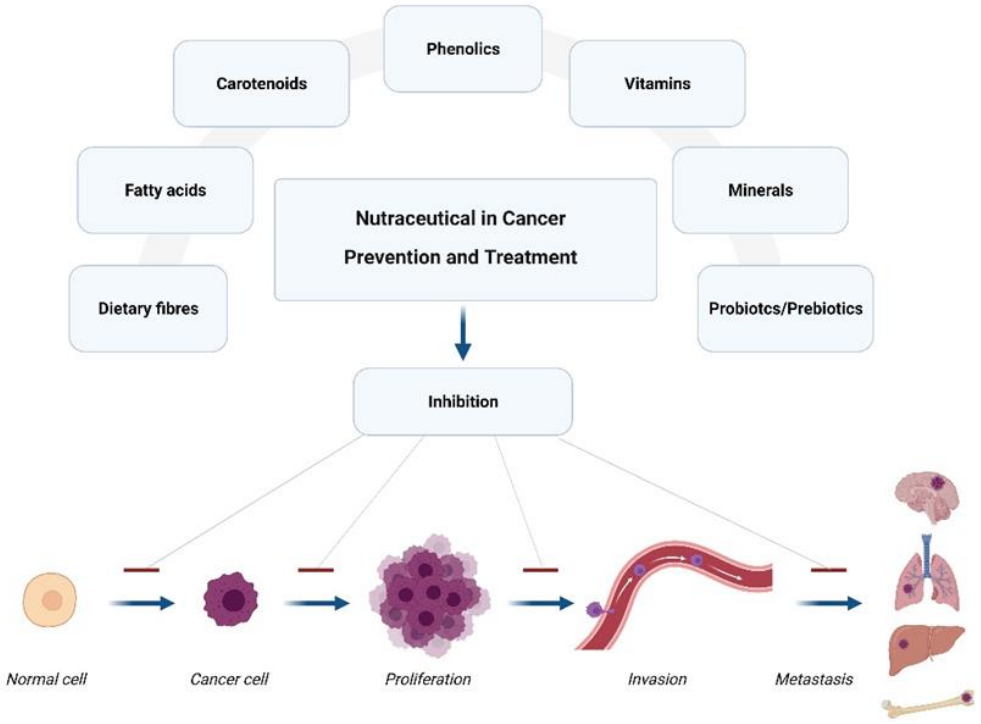


Figure 4. *Schematic illustration of various kinds of nutraceuticals used in cancer prevention and treatment.*

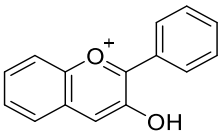
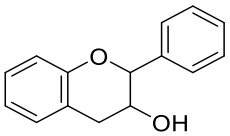
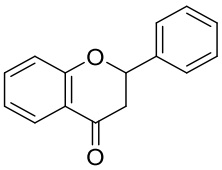
Many species of plants and fruits of the Mediterranean area have shown a high onconutraceutical potential thanks to their bioactive compounds action. In onconutraceuticals, particular attention is paid to dietary polyphenols and carotenoids

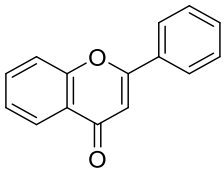
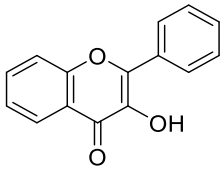
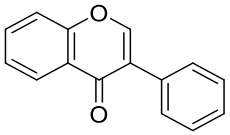
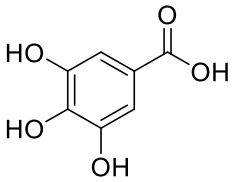
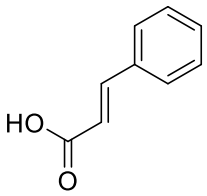
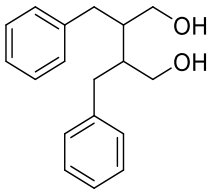
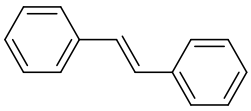
which represent the primary source of bioactive compounds with potential antioxidants and anticancer properties in human nutrition.

1.2. Polyphenols

In the multiplicity of phytochemicals, polyphenols represent the largest group with numerous different chemical structures, and more than 8000 have been described. Polyphenols are compounds having at least one aromatic ring functionalized with one or more hydroxyl groups. Natural polyphenols are a large group of secondary metabolites in plants and include small molecules and highly polymerized compounds that protect plants from disease, infection and damage (Fu et al., 2010, Deng et al., 2013). Polyphenols are widely used in foods and drinks of plant origin. Depending on the interconnection and the number of phenolic rings, polyphenols are collected in different main classes, phenolic acids, lignans, stilbenes and flavonoids, with phenolic acids and flavonoids representing respectively 30% and 60% of the total diet polyphenols (Table 1) (Marino et al., 2023).

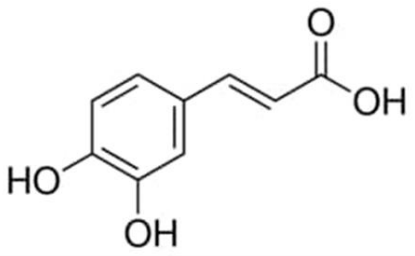
Table 1. *Polyphenol and secondary metabolite classification and dietary sources.*

Classification		Structure	Major Dietary Sources
Flavonoids	Anthocyanins		Berries, grapes, cherries, plums, currant, pomegranates, red cabbage
	Flavanols		Apples, pears, legumes, tea, cocoa, wine
	Flavanones		Citrus fruits

	Flavones		Parsley, celery, orange, onions, tea, honey, spices, oregano
	Flavonols		Berries, apples, broccoli, beans, tea, asparagus, leafy greens, onion
	Isoflavones		Soy
Phenolic acids	Hydroxybenzoic acids		Pomegranate, grapes, berries, guava, blackcurrant, walnuts, chocolate, wine, green tea
	Hydroxycinnamic acids		Coffee, cereal grains, tea leaves, red ones
	Lignans		Flaxseeds, sesame, barley, buckwheat, oats
	Stilbenes		Grapes, berries, red wine

Phenolic acids include two main classes, those deriving from benzoic acid and those deriving from cinnamic acid. The benzoic acid content in edible plants is generally very low. Examples of hydroxybenzoic derivatives are gallic, p-hydroxybenzoic, vanillic, and syringic acids, whereas caffeic, ferulic, sinapic, and p-coumaric acids belong to hydroxycinnamic acids. Ellagic acid, abundant in cranberries, strawberries, blueberries,

and blackberries, has been shown to decrease blood pressure and high blood cholesterol, to exert anti-inflammatory properties and even to reduce skin wrinkles from radiation. Another example is gallic acid, which can be found in tea, mango, rhubarb, and soy and which is known mainly for its antioxidant effect (Roche et al., 2017). The hydroxycinnamic acids are present at high concentrations in many food products, including fruits (especially the red-colored ones), vegetables, tea, cocoa, wine, tea leaves, coffee, and whole grains. Caffeic and quinic acids combine to form chlorogenic acid present in many types of fruit and in high concentrations in coffee (70-350 mg per single cup). Caffeic acid (Figure 5), in both free and esterified forms, is generally the most abundant phenolic acid and represents between 75% and 100% of the total content of hydroxycinnamic acids of many fruits (Durazzo et al., 2019).



Caffeic acid

Figure 5. *The chemical structures of caffeic acid.*

Lignans are vascular plant secondary metabolites, with widespread occurrence in the plant kingdom. Lignans are diphenolic compounds derived from the combination of two phenylpropanoid (Figure 6) and to which are attributed a wide range of physiological properties, positively influencing human health. The main sources of dietary lignans are oilseeds (i.e., flax, soy, rapeseed, and sesame), whole-grain cereals (i.e., wheat, oats, rye, and barley), legumes, various vegetables, and fruit (particularly berries), as well as beverages, such as coffee, tea, and wine, and, recently, lignans are also reported in dairy

products, meat, and fish. The main dietary lignans are secoisolariciresinol, matairesinol, lariciresinol, pinoresinol, medioresinol, and syringaresinol. The intake of lignans has been mostly related to their possible cancer chemopreventive actions (due to their phytoestrogen properties) and in the prevention of cardiovascular diseases (Durazzo et al., 2018).

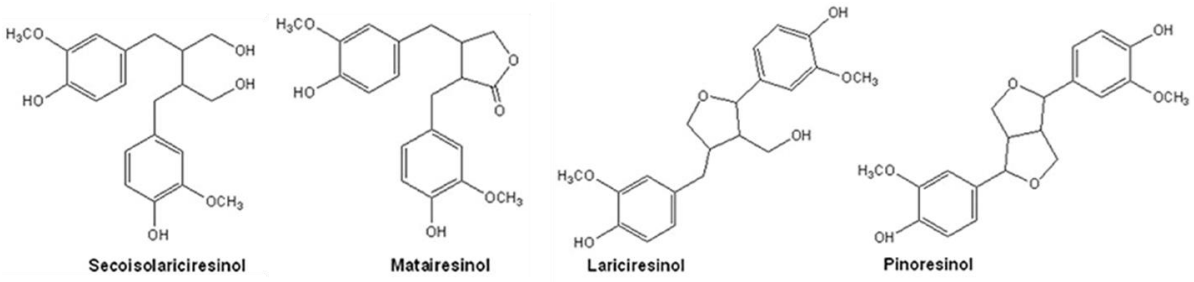


Figure 6. *The chemical structures of some lignans.*

The basic chemical structure of stilbenes consists into two benzene rings linked by a double bond, the E isomer being the most common configuration. Stilbenes are reported to be present in grapes, almond, bean, blueberries, bilberries, peanuts, grapevine, cranberries, mulberries, plum, and wine. Stilbenes present a high diversity in their phenolic structures (various chemical substituents and polymerization), which is a determining factor for their absorption and metabolism rates (El Khawand et al., 2018). Stilbenes intake has been associated to a reduced all-cause mortality as well as to a reduced the risk of hypertension onset, diabetes, and obesity. The most known and studied among stilbenes is resveratrol (Figure 7), a compound present in nature in the two isomeric forms *cis* and *trans*, with the most common trans form and with greater biological activity. It was first identified in the *Vitis vinifera* wine grape in 1976, and today it has been found in more than 70 plant species. *Polygonum cuspidatum*, a herb

used in traditional Chinese and Japanese medicines, is one of the major sources of resveratrol (Durazzo et al., 2019).

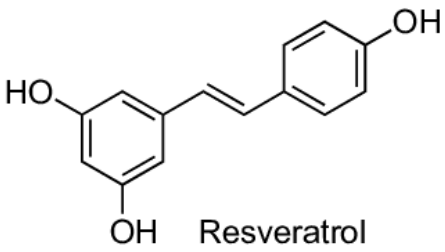


Figure 7. *The chemical structures of resveratrol.*

Flavonoids are the largest class of naturally occurring polyphenols; more than 5,000 have been described. They have a common structure consisting of two aromatic rings (A and B) and a benzopyran ring (C) (Figure 8), they can in turn be divided into 6 subclasses according to the type of heterocycle involved: flavonols, flavones, isoflavones, anthocyanidins and flavanols (catechins and proanthocyanidins). In most of the cases, three or more –OH groups are linked to their backbone structure. Flavonoids can occur as aglycones or as conjugated to sugars and/or organic acids. The degree and pattern of hydroxylation, prenylation, alkalization, or glycosylation reactions modify the primary structure of the molecule (Khoddami et al., 2013). The substitution of chemical groups in the flavonoid structures is correlated to the corresponding biological and/or chemical properties and bioavailability (Teng and Chen, 2019). A wide range of pharmacological activities, including antioxidant, antibacterial, hepatoprotective, anti-inflammatory, and antihyperlipidemic effect, are attributed to flavonoids (Abenavoli et al., 2018, Riccio et al., 2018).

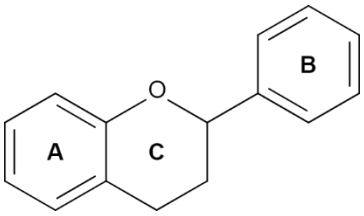


Figure 8. *The basal structures of flavonoids.*

The most abundant flavonoids in the plant kingdom are flavonols. Flavonols are reported to be present in onions, apples, persimmon, saffron, berries, broccoli, lettuce, tea, and red wine. Flavonols accumulate in external tissues, as their biosynthesis is stimulated by light. The flavonol contents vary in fruits on the same plant, and even on the same piece of fruit, this is due to the light exposure to their different edges. Quercetin and kaempferol are the main representative molecules (Figure 9). Quercetin is found mainly in onions, apples and berries, it is usually present in the form of O-glycoside, therefore more than 170 different quercetin-glycosides have been identified, although D-glucose is the most frequent sugar residue (Saraf et al., 2007). Quercetin has attracted the attention of researchers for its cancer prevention activity, reduction of chronic inflammation and cardiovascular disease. The estimated mean intakes of quercetin is 29.4 mg per day (Ranka et al., 2008). Evidence suggests that quercetin negatively regulates key signaling pathways associated with life-threatening diseases, such as NF-κB, MAPK, PI3K-AKT, and mTOR. Quercetin is also marketed as a dietary supplement, and it is suggested to be assumed a dose up to 1,000 mg/daily, an amount that exceeds the usual dietary intake levels (Andres et al., 2018). Kaempferol is a natural flavonol present in different edible plants, such as tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries, and grapes. It has been described to possess anti-inflammatory, anticancer, and notably cardiovascular protective properties (Devi et al., 2015, Rajendran et al., 2014).

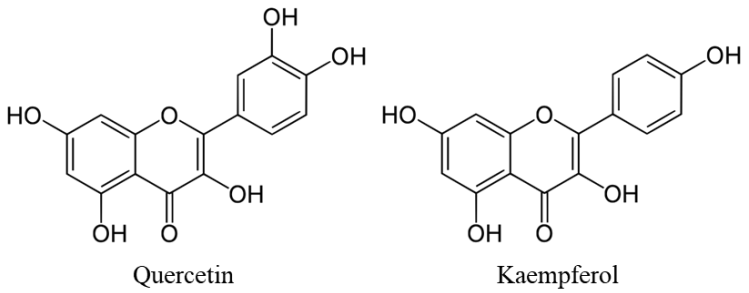


Figure 9. *The chemical structures of Quercetin and Kaempferol.*

Anthocyanins are a widespread class of phenolic compounds characterized by strong and broad absorption in the UV-Visible region, responsible for the intense red-orange to blue-purple color found in fruit, flowers and vegetables, which makes them the most important group of visible plant pigments besides chlorophyll. Anthocyanins are water-soluble, non-toxic flavonoids structurally characterized by a polyphenolic heteroaromatic C15 three ringed skeleton. Anthocyanins are glycosylated analogues (mainly in the C3 position) of anthocyanidins, both based on the basic structure of the chromophore 2-phenyl-benzopyril (flavylium ion) having glucose as the most common sugar attached; however, rhamnose, xylose, galactose, arabinose and rutinose can also be found (Pervaiz et al., 2017). Common anthocyanins are cyanidin, delphinidin, malvidin and peonidin (Figure 10). The major sources of anthocyanins are black currant, red raspberry, elderberry, strawberry, plum, pomegranate, blood oranges, beans, cabbage and red onions (Weber and Larsen, 2017).

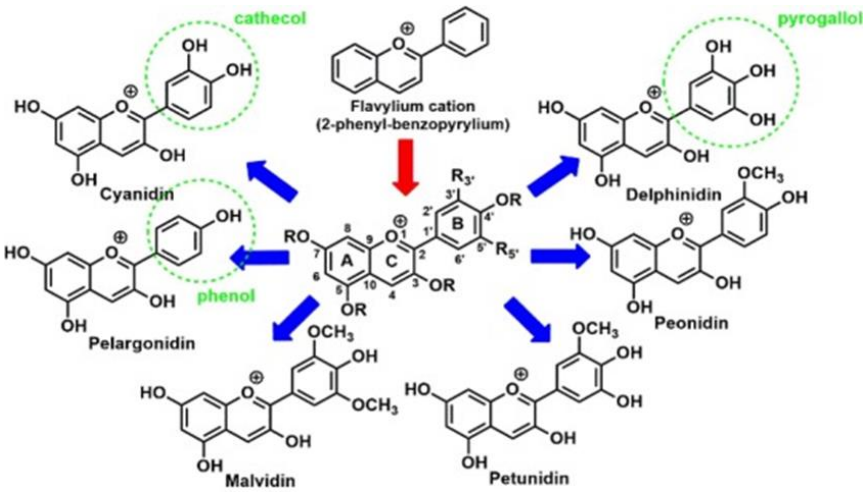
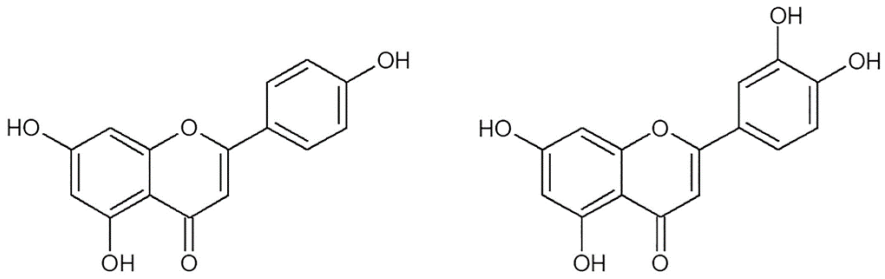


Figure 10. *Chemical structure of some anthocyanins.*

Anthocyanins increase the antioxidant response of plants, assisting the correct physiology of tissues. Numerous studies have suggested that anthocyanins possess numerous health-promoting properties, showing antidiabetic and anti-obesity effects and act as neuroprotective agents (Pojer et al., 2013). In addition, they appear capable to reduce inflammations and recover from vision complications, as well as to protect from cardiovascular risks, to inhibit cancer growth, to encourage several antimicrobial benefits, and as seen for plants they may prevent or delay some types of cell damage. Anthocyanins are renowned for their antioxidant properties, which are closely related to their molecular structure and redox properties. All major anthocyanins have good antioxidant properties compared to vitamin E; in particular delphinidin probably represents the best antioxidant among the anthocyanins (Sinopoli et al., 2019). Although anthocyanins experimentally inhibit cell growth, induce cell cycle arrest, stimulate apoptosis (or autophagy), and exerts anti-invasion and anti-metastatic actions, there are conflicting clinical results concerning the possible intake of anthocyanins and cancer prevention in humans (Lin et al., 2017).

Flavones are much less common than flavonols in fruits and vegetables. They are found mainly as luteolin and apigenin glycosides (Figure 11). Examples of food sources

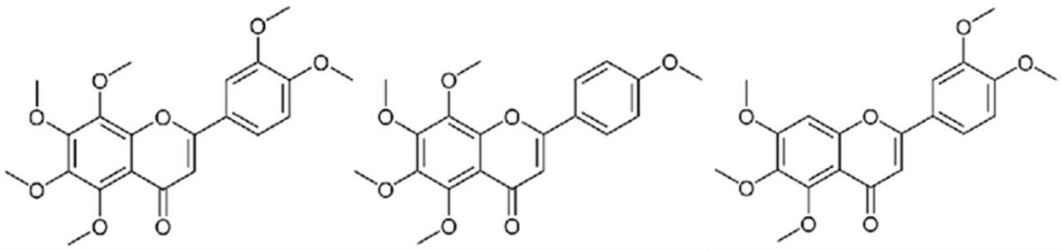
are parsley, celery, acerola, apricot, cashew, beans, cabbage, thistle, dandelion, apple, artichoke, mango, papaya and onion (Siriamornpun and Kaewseejan, 2017).



(a) apigenin (b) luteolin

Figure 11. *The chemical structures of (a) apigenin and (b) luteolin.*

Citrus peel contains large quantities of polymethoxylated flavones: tangeretin, nobiletin and sinensetin, which appear to be the flavonoids with the greatest hydrophobic character (Figure 12) (Bataglion et al., 2015).



Nobiletin Tangeretin Singeretin

Figure 12. *Chemical structure of some polymethoxylated flavones.*

There is evidence that dietary flavones intake may help reduce weight gain over time in population (Adriouch et al., 2018). Furthermore, a systematic review of epidemiological studies retrieved a cohort study in which a significant reduction between ovarian cancer incidence and luteolin intake was observed (Mohammadi et al., 2016). Health-promoting and therapeutic beneficial effects, that is, on diabetes, amnesia,

Alzheimer's disease, depression, insomnia, and cancer related to apigenin have been recently reported with reference to in vivo research, chemistry, and nutraceutical features (Salehi et al., 2019b).

More than 160 naturally occurring flavanones belonging to 36 plant families have been described. Flavanones are present in many vegetables and spices, such as tomato, potato, rosemary and peppermint, and in high concentrations in citrus fruits (Bajkacz and Adamek, 2018). Flavanones are generally glycosylated by a disaccharide at position 7, either a neohesperidose which imparts the bitter taste (example: with naringenin in grapefruit), or a rutinose which is tasteless. The main aglycones are naringenin, hesperetin and eriodictyol. In particular, hesperetin and its derivatives are characteristic flavanones of sweet orange, tangelo, lemon and lime, naringenin and its derivatives are characteristic of grapefruit and sour orange, while eriodictyol of lemons (Barreca et al., 2017). A glass of orange juice contains between 40 and 140 mg of glycosylated flavanones. Figure 13 shows the chemical structures of the molecules belonging to the flavanones.

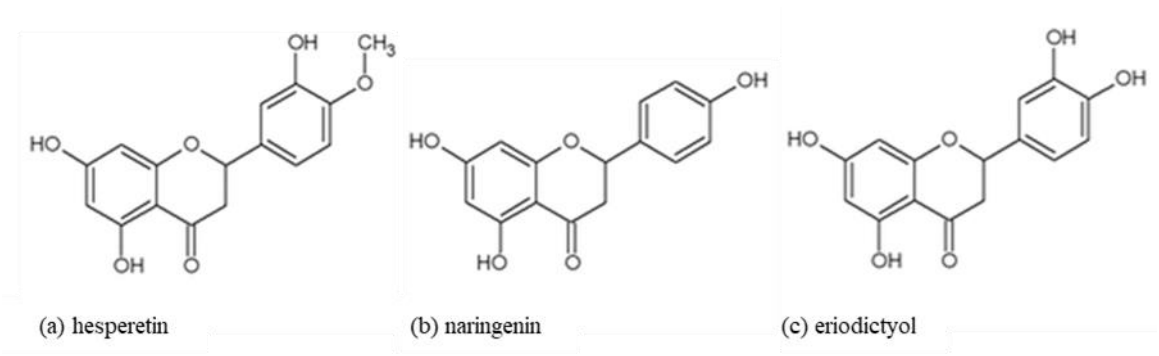


Figure 13. *The chemical structures of (**a**) hesperetin, (**b**) naringenin and (**c**) eriodictyol.*

Flavanone intake has been associated with a reduced risk of diabetes and obesity (Adriouch et al., 2018). Naringenin is one of the best studied of the flavanones and numerous articles have highlighted its potential use against oxidative stress,

inflammation, neurological disorders and in particular cardiovascular/metabolic diseases (Salehi et al., 2019a). Epicatechins and catechins belong to the subgroup of monomeric flavanols, while proanthocyanidins belong to the subgroup of polymeric flavanols. The most common flavanols in nature are (+)-catechin and (-)-epicatechin (Bernatoniene and Kopustinskiene, 2018). Flavanols can form gallic acid conjugates such as epicatechin gallate and epigallocatechin gallate (Figure 14) (Braicu et al., 2013).

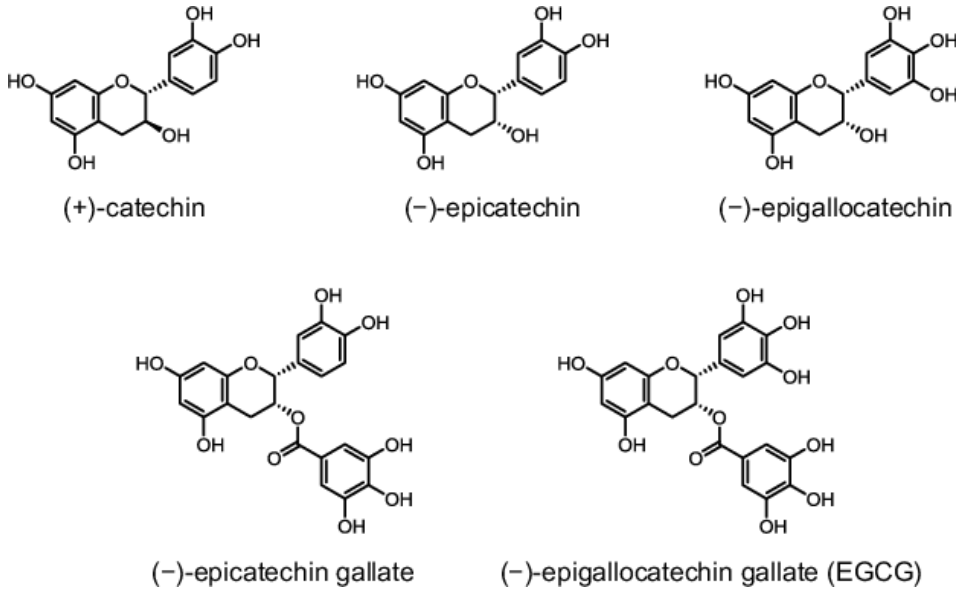


Figure 14. *Chemical structures of the most common flavanols in nature.*

Catechins are found primarily in plums, apples, strawberries, cherries, lentils, chocolate and beans, with a maximum observed concentration of 49 mg/100 g in plums and 157 mg/100 g in large beans. They are also present in grapes, while procyanidins are found in the seeds and skin. Catechins are the major polyphenols in tea and have been related to the beneficial health effects of tea (Annunziata et al., 2018, Zhang et al., 2018). Epigallocatechin-3-gallate (EGCG) is abundant in tea, whose consumption has been correlated with a low incidence of chronic cardiovascular disease and cancer (Peluso and Serafini, 2017). Cocoa products, such as chocolate, are rich in catechin and epicatechin

(Rodríguez-Carrasco et al., 2018). Cocoa and chocolate flavonols have attracted clinical attention for the prevention of cardiovascular and metabolic diseases. The use of flavanol-rich cocoa products lead to a reduction in blood pressure (Ried et al., 2012). The anti-obesity potential of flavonols has also been explored. Flavanol intake has been shown to reduce body mass index and waist circumference (Akhlaghi et al., 2018). Furthermore, cocoa flavonol intake may improve vascular function, reduce exercise-induced oxidative stress, and alter fat and carbohydrate utilization during exercise, but without affecting exercise performance (Decroix et al., 2018).

Isoflavones (Figure 15) are compounds structurally similar to estrogens. Although they are not steroids, they have hydroxyl groups at the 7 and 4' positions in a spatial configuration analogous to those found in estradiol. This characteristic confers pseudohormonal properties to isoflavones, including the ability to bind to estrogen receptors; for this reason they are classified as phytoestrogens (Anandhi Senthilkumar et al., 2018). The main representative components are genistein, daidzein, biocanin A and glycitein. Isoflavones are found almost exclusively in the legume family such as soybeans, but also in some fruits such as apple, apricot, blackcurrant, cherry, plum (Abrankó et al., 2015, Bustamante-Rangel et al., 2018).

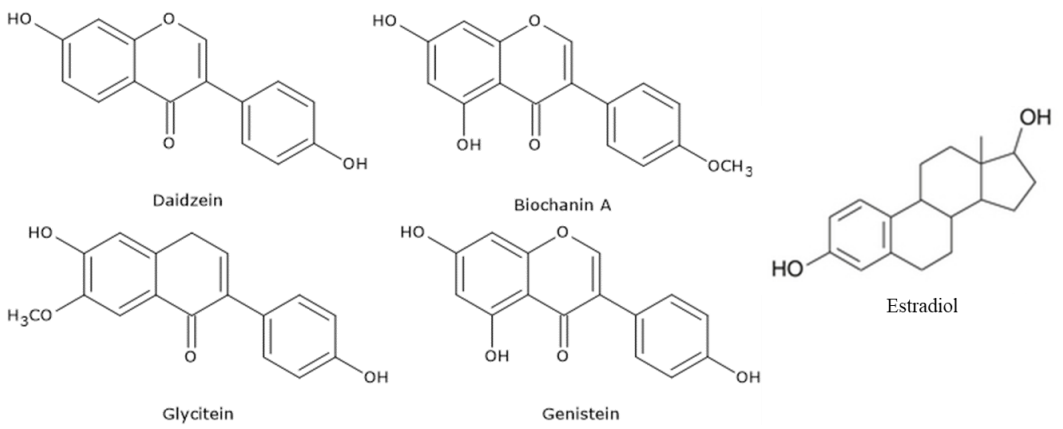


Figure 15. *Chemical structure of some isoflavones and estradiol.*

Numerous studies show that phytoestrogens can have a beneficial, albeit modest, effect on menopausal symptoms. Furthermore, there is preliminary evidence that taking phytoestrogens could reduce the incidence of prostate and breast cancer (Andrew and Izzo, 2017, Fritz et al., 2013, van Die et al., 2014).

1.2.1. Biological Activity of Natural Polyphenols

The biological activity of polyphenols is determined by several factors, the most important are:

- direct interaction with receptors and enzymes involved in signal transduction which can modify the redox state of cells and initiate a series of redox-dependent reactions;
- interactions with estrogen receptors with consequent effects on endocrine functions. One of the main examples is represented by the interaction of soy isoflavones with estrogen receptors which explains their role in the prevention of the main manifestations of menopause;
- the well-known antioxidant and free radical scavenger activity with which cells are protected from oxidative damage;
- pro-oxidant activity which can induce apoptosis of tumor cells and prevent the development of tumors.

Polyphenols have shown numerous biological activities and health benefits, for the prevention and treatment of diseases, among the most important are the antioxidant, cardioprotective and anticancer properties.

1.2.1.1. Antioxidant Activity

The best described property of polyphenols is their antioxidant capacity against free radicals normally produced by cellular metabolism or in response to external factors.

Free radicals are molecules or molecular fragments containing one or more unpaired electrons in the outermost atomic or molecular orbital. Under physiological conditions, living systems have endogenous defense systems that protect structural and functional biomolecules from attack by free radicals. These defense systems, which can be of the enzymatic type (glutathione, superoxide dismutase, catalase) and non-enzymatic (antioxidant molecules and vitamins taken with the diet), react with the radical species before they can attack the biological structures, damping their potential harmful. In the absence of this "antioxidant barrier", free radicals and reactive oxygen species (ROS) react rapidly with life-critical biomolecules such as DNA, lipids and proteins, causing severe cellular damage and even cell death. A situation of oxidative stress is therefore triggered, which is responsible for important damages that compromise the functionality of cells and tissues (Di Meo and Venditti, 2020).

Agents with a negative reduction potential capable of supplying free radicals and ROS with the electrons they lack, restoring the chemical balance of the system in which they act, are considered "antioxidants". A prerequisite to act as antioxidants is that, once oxidized, their radical form is non-reactive or not very reactive towards the other molecules. Antioxidants exert their action at rather low concentration levels; when these concentrations increase, some of them can become pro-oxidants and favor the formation of radicals (Rudra et al., 2021).

There are two main ways to obtain antioxidant agents; in the first place the intake of natural antioxidants from fresh and dried fruit and vegetables, in fact most of the known antioxidants belong to the vegetable kingdom. A second way to make new antioxidants

available is the synthetic one. Contrary to natural ones, synthetic antioxidants are developed and produced for the exclusive benefit of mankind; their safety is tested with standardized procedures to protect the consumer against possible health risks, although some studies indicate that they are not entirely safe (Carocho and Ferreira, 2013). On the contrary, natural antioxidants, such as polyphenols, have important free radical inhibitory capabilities but do not have important harmful, toxic and dangerous effects (Gulcin, 2020). The antioxidant activity of phenolic compounds is due to the presence of hydroxyl groups linked to the aromatic structures and the geometry of the molecule. Phenols are multifunctional antioxidants. They can act as reducing agents, hydrogen-donating antioxidants, singlet oxygen quenchers, and metal cation chelating agents. Flavonoids inhibit nitric oxide synthase which generates nitric oxide, which in turn reacts with free radicals to generate the peroxynitrite species, as well as being a radical itself. Xanthine oxidase is implicated in oxidative damage because it reacts with molecular oxygen and releases superoxide. Flavonoids, particularly quercetin and luteolin, are potent inhibitors of xanthine oxidase (Leopoldini et al., 2011).

1.2.1.2. Anticancer Activity

Cancer is a leading cause of death worldwide. The anticancer effects of polyphenols have been observed in the mouth, stomach, duodenum, colon, liver, lung, mammary gland or skin. Many polyphenols were tested, such as proanthocyanidins, flavonoids, resveratrol, tannins, epigallocatechin-3-gallate, gallic acid and anthocyanins; all showed protective effects in some models, although their mechanisms of action were found to differ through anti-initiating, anti-promoting, anti-progression, and anti-angiogenesis actions, as well as modulating the immune system (Erices et al., 2018, Rengasamy et al., 2019).

Polyphenols can intervene on all stages of carcinogenesis with various mechanisms of action. In fact, they can act as a scavenger to block carcinogenic agents (especially free radicals) or arrest the cell cycle through the action that some of them exert on the production of key proteins or induce apoptosis of tumor cells or exert angiogenic action. The potential anticancer effect of polyphenolic compounds also appears to be specific to cancerous cells; the cytotoxicity of phenols on cancer cell lines appears to be important while on normal cells they have an insignificant or low effect (Li et al., 2014).

Furthermore, the anticancer actions of polyphenols involve redox changes, enzyme modulation, and signaling kinases resulting in effects on multiple genes and cellular signaling pathways (Maru et al., 2016, Mileo and Miccadei, 2016). Isoflavone intake was significantly associated with a reduced risk of lung and stomach cancer and, to a lesser extent, breast and colorectal cancers (Grosso et al., 2017).

There is some evidence that isoflavones may possibly reduce the risk of certain types of cancer (e.g., colorectal, breast, and prostate cancer), particularly in Asian countries (Rothwell et al., 2017). The mechanism of the antitumor effects of flavonoids appears to depend on their structure, with each compound exhibiting various biological potencies and mechanisms of action. However, the essential feature of flavonoids is their free radical scavenging activity, which is partially responsible for their anticancer effects. Flavonoids have antiproliferative effects and induce apoptosis in several cancer cell lines. As a free radical scavenger, flavonoids inhibit invasion and metastasis (Leopoldini et al., 2011).

Resveratrol could inhibit each stage of multistage carcinogenesis, eliminate incipient populations of androgen-dependent prostate cancer cells through androgen receptor antagonism, and eliminate incipient populations of androgen-independent prostate cancer cells by short-circuiting factor-dependent autocrine circuits of epidermal growth in cancer

cells. Resveratrol is a prototype of a plethora of bioactive polyphenols in the food supply that has just begun to be mined for cancer preventive agents. The identification of resveratrol as a cancer preventive agent is largely due to its high abundance in nature. For example, polyphenolic fractions of grape seeds have recently been shown to potently antagonize chemical carcinogenesis, and resveratrol accounts for 5%-10% of grape skin biomass. Therefore, resveratrol may represent the tip of the iceberg of a large class of stilbene and related polyphenolic natural products for cancer prevention (Stewart et al., 2003).

1.2.1.3. Cardioprotective Activity

A large number of naturally occurring compounds and foods are promoted for the prevention of cardiovascular disease (Tapsell et al., 2019). Consequently, numerous studies have explored the possible association between the intake of foods rich in polyphenols (for example, beverages such as cocoa, fruit and vegetables, tea, extra virgin olive oil and wine) and cardiovascular disease. The association between the intake of total polyphenols and subgroups of polyphenols and the risk of cardiovascular events revealed a 46% reduction in the risk of cardiovascular disease. The polyphenols with the strongest inverse associations were flavanols, lignans, and hydroxybenzoic acids (Tresserra-Rimbau et al., 2014).

Interactions between platelets and blood vessels are implicated in the development of thrombosis and atherosclerosis. Flavonoids inhibit platelet aggregation and adhesion; through different biochemical mechanisms they appear to influence different pathways involved in platelet function. Indeed, flavonoids appear to increase vasodilation by inducing vascular smooth muscle relaxation which may be mediated by inhibition of protein kinase C, PDEs or by decreasing cellular calcium uptake.

An increase in polyphenol intake was associated with a decrease in inflammatory biomarkers (i.e., vascular cell adhesion molecule, intercellular adhesion molecule, interleukin, tumor necrosis factor alpha, and monocyte chemoattractant protein), with a positive effect on blood pressure and the lipid profile (Medina-Remón et al., 2017).

Although it is not fully understood how polyphenols exert their protective effects, it is believed that the modulation of nitric oxide production and the induction of antioxidant defenses may play an important role (Costa et al., 2017).

1.2.2. Bioavailability

A limitation in the use of polyphenolic compounds in disease prevention is due to their bioavailability. The common definition of bioavailability was the proportion of the nutrient that is digested, absorbed and metabolized through normal pathways (Manach et al., 2004). Generally, after oral ingestion, polyphenols are present in low concentration in the blood and urine, as they undergo metabolic changes and steps. In fact, they initially undergo a first enzymatic modification and a metabolic process by the intestinal microflora, subsequently are primarily metabolized in the liver by methylation, glucuronidation and sulfation, and are rapidly excreted in bile and urine (Figure 16). Only a small part reaches the organs and tissues (Table 2) (Marino et al., 2023).

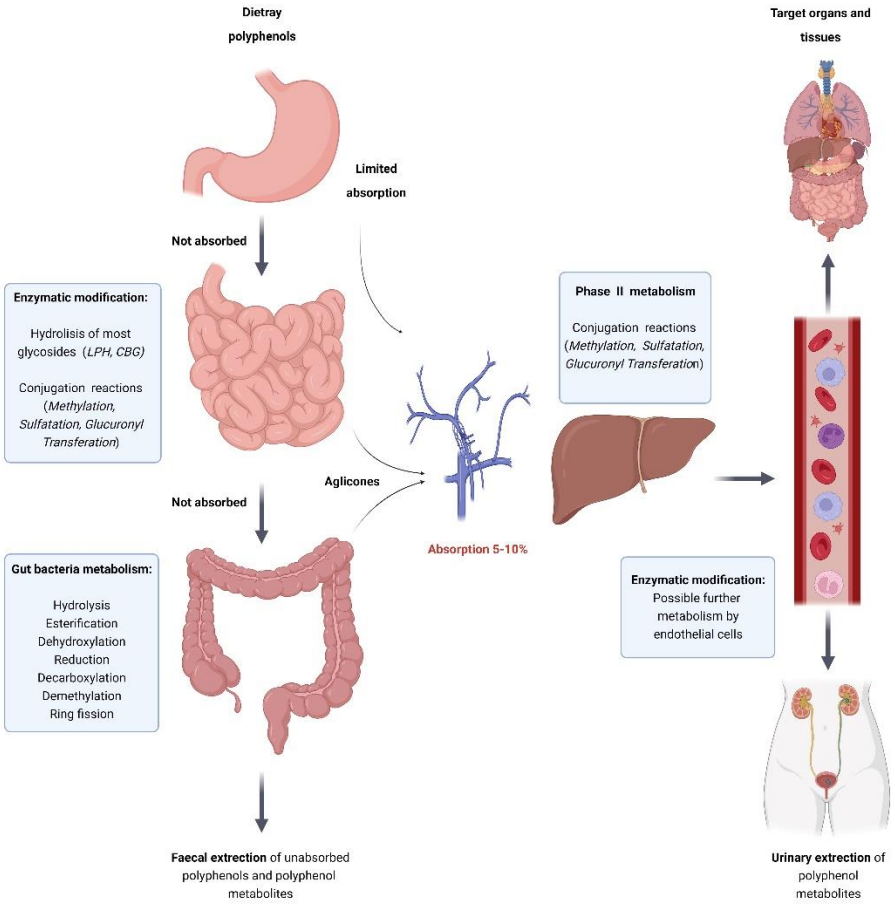
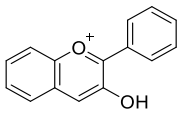
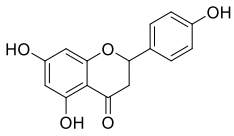
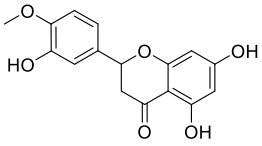
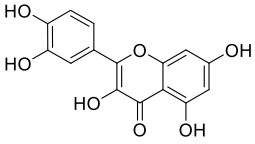
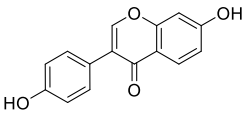
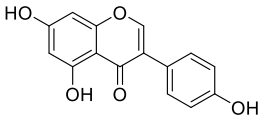
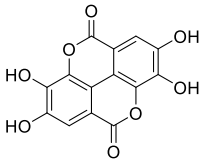
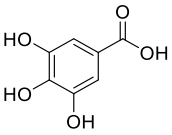


Figure 16. *Schematic representation of the absorption and metabolism of food polyphenols.*

Table 2. *Bioavailability in human of some natural polyphenols.*

Polyphenol	Structure	Food	Concentration in food	Plasma Concentration
Anthocyanins		Black berries	960 µM 200 g^{-1}	NA
Naringenin		Orange	7.9 mg 100 g^{-1} FW	Mean Cmax 80 nM, Tmax 5.88 h
Hesperetin		Orange	53.1 mg 100 g^{-1} FW	Mean Cmax 90 nM, Tmax 7 h
Quercetin		Dry shallot skin	4.9 µmol g^{-1} FW	Mean Cmax 3.95 µM, Tmax 2.78 h

Daidzein		Soy milk	2.2 mg 100 mL^{-1}	196.1 nM after 5 days
Genistein		Soy milk	6.8 mg 100 mL^{-1}	797.04 nM after 5 days
Ellagic acid		Black raspberry	300 µg g^{-1} DW	Mean Cmax 10 nM, Tmax 1.98 h
Gallic acid		Grape skin	700 µg g^{-1} DW	NA

FW: fresh weight; DW: dry weight; NA: not available

Their limit and speed of absorption in intestines is decided by their chemical structure. The aglycones could be absorbed from the small intestine. However, most polyphenols were presented in food in the form of esters, glycosides, or polymers that cannot be absorbed in their native form. Essentially, the forms reaching the blood and tissues are different from those present in food.

Green tea contains catechins with high bioavailability, and citrus fruits are a rich source of flavanones (Srinivasan, 2005). When the concentrations of phenolic compounds are measured from plasma and urine, the antioxidant potential of plasma, after ingestion of a polyphenol rich food, provides the evidence needed for quantifying the absorption of such compounds in the intestines (Natsume et al., 2003). The plasma content gradually decreases when a number of flavonoids are absorbed into the intestines. Plasma albumin has a high affinity, this is the reason that quercetin has a relatively higher elimination half-life. Polyphenols generally contain one sugar but may have two or three, and substitutions of a wide range for sugars. The glucose and rhamnose are chemically linked and sometimes xylose, glucuronic acid, and galactose are as well (Zhang et al., 2006).

Chemical, physical, and biological attributes of polyphenols are decided by glycosylation and this is the reason for the difference in hydrophilic property of quercetin and quercetin-3-O-rhamnoglucoside. Gastrointestinal mucosa and colon micro flora are the sites where the activity of glycosidase can occur. β-glucosidases are sometimes secreted by human cells, and particularly the tissue-specific model of expression is usually regulated while the development is being done. When xylose or glucose is attached with polyphenols, these are the latent substrates for individual cells.

Microflora-rhamnosidases is uniquely able to cleave polyphenol bound to rhamnose because it cannot be cleaved by human β-glucosidases. Epicatechins are mostly acylated with acids like gallic acid. Irrespective of hydrolysis, flavanols are absorbed while passing through the biological membranes (Purushotham et al., 2009). Sugars, lipids, and organic acids are generally esterified with caffeic acid. When quinic acid is esterified with caffeic acid, they form chlorogenic acid which is found in significant amounts in coffee. Chlorogenic acid cannot be cloven by esterases present in human tissues and caffeic acid cannot release from chlorogenic acid. The only site where chlorogenic acid can be metabolized is within the colon microflora.

Furthermost, polyphenols pass through the small intestine without being absorbed, thus manipulating the intestinal microbiota that colonize there (Kim et al., 2016). First, polyphenols are biologically transformed into their relatively more bioavailable metabolites. And second, polyphenols modify the configuration of the gut microbial community most likely by the inhibition of pathogenic bacteria and the stimulation of beneficial bacteria. In the latter, these may act as a prebiotic metabolite and enrich the beneficial bacteria. Therefore, the interactions of dietary polyphenols and gut microbiota may result in impact on human host health (Klinder et al., 2016).

1.3. Carotenoids

Carotenoids are a family of compounds of over 600 lipophilic plant pigments, and therefore are hydrophobic molecules with very low water solubility that function in the hydrophobic areas of the cell. Their structure is based on a skeleton of 40 carbon atoms formed by the union of eight covalently bonded isoprene units which can be linear or modified to have, in one or both ends, cyclic structures carrying different functional groups (Maoka, 2011). This results in a conjugated hydrocarbon backbone by alternating double and single bonds. Double bond conjugation is related to photochemical properties and chemical reactivity to molecules, including free radicals.

Carotenoids may exist in different configurations due to isomerism around the C-C bonds, obtaining trans and cis isomers. Isomers differ not only in their melting points, solubility, and stability but also in ultraviolet characteristics (Gong and Bassi, 2016).

Carotenoids can be found in free form or esterified with organic fatty acid such as palmitic, estearic, linoleic. The natural functions and properties of carotenoids depend on the molecular structure. The conjugated polyene chromophore presented in carotenoid molecule determines the light absorption and light-harvesting properties. Therefore, chromophore is the part of a carotenoid molecule responsible for its color and photoprotective actions. The wavelengths of the colors of the carotenoids fall between 400 and 600 nm (Jomova and Valko, 2013).

These lipophilic compounds are responsible for the yellow, orange, and red colors in many fruits and flowers without chlorophyll and for the coloring of many species such as plants, fungi, algae and bacteria. Lycopene, β-carotene, α-carotene, lutein, zeaxanthin, and β-cryptoxanthin are the most common carotenoids in human serum (Figure 17) (Shardell et al., 2011). Carotenoids can be classified into two groups: xanthophylls, including lutein and zeaxanthin, and carotenes, such as β-carotene, α-carotene and

lycopene. As antioxidants, carotenoids are generally sensitive to light, oxygen and heat, which can lead to storage and handling difficulties. Xanthophylls, which are just hydrocarbons, are relatively hydrophilic compounds due to the presence of hydroxyl groups and ketone groups at the end rings.

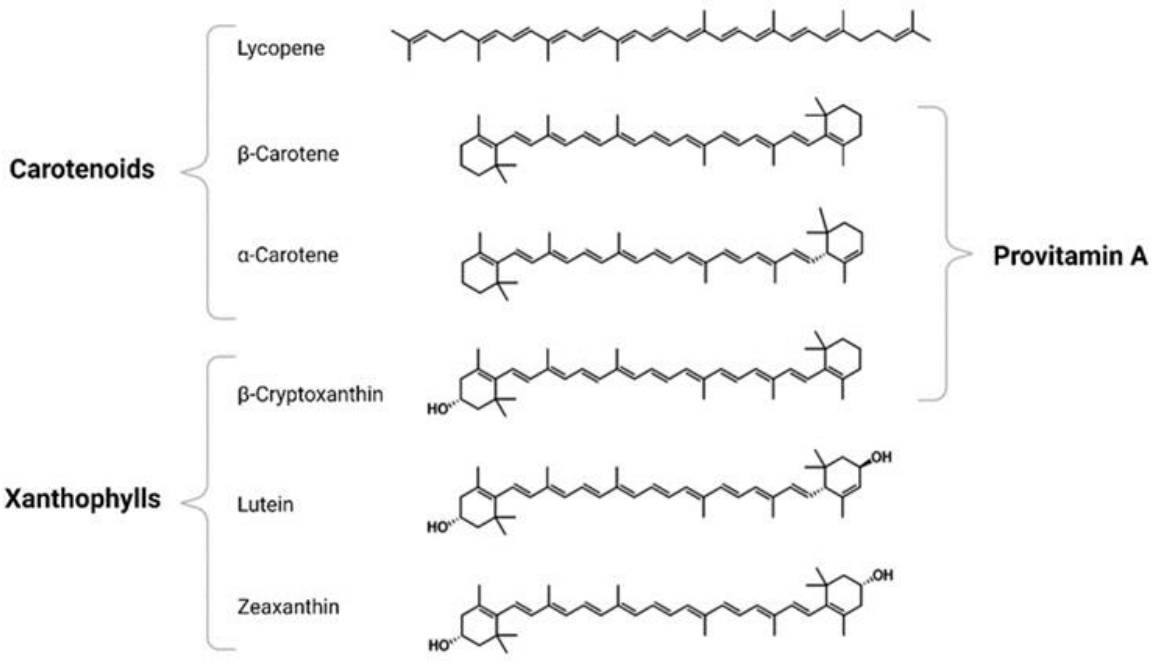


Figure 17. *Structure of the most important carotenoids.*

The carotenoid content in fruits and vegetables depends on various factors such as harvest, climatic conditions, variety, ripening stage, processing and storage conditions (Table 3). Common carotenoids sources are brightly colored fruits and vegetables. The main food sources of dietary lycopene are mainly tomatoes, tomato products and fruits such as watermelon. β-carotene is found in orange and yellow fruits and green leafy vegetables such as carrots, mangoes and kale. Like β-carotene, α-carotene is found in orange and yellow fruits and green leafy vegetables such as pumpkins, carrots and tomatoes at lower levels. Lutein and zeaxanthin are found in a variety of vegetables and egg yolk with the kale, spinach and broccoli containing the highest content. β-

cryptoxanthin can be found in a variety of dietary sources such as papaya, pumpkins, egg yolk, butter, and apples (Rowles III and Erdman Jr, 2020).

Table 3. *Carotenoids content per portion (mg).*

Food	Pz (g)	Lyc (mg)	Food	Pz (g)	β-Car (mg)	Food	Pz (g)	α-Car (mg)	Food	Pz (g)	β-Cry (mg)	Food	Pz (g)	Lut-Zea (mg)
Tomato juice, 100%	248	22.4	Carrot juice	240	22.3	Carrot juice	240	10.4	Persimmon	168	2.4	Cooked Spinach	190	29.7
Spaghetti sauce	130	16.5	Baked sweet potato	150	17.2	Cooked pumpkin	245	6.6	Papaya	304	1.8	Cooked Kale	130	25.4
Watermelon	286	13.0	Cooked Spinach	190	13.7	Carrots	110	3.8	Mandarin oranges, canned	189	1.5	Dandelion greens	55	7.5
Canned Stewed tomatoes	255	10.4	Cooked Kale	130	11.4	Cooked winter squash	245	1.7	Red peppers	119	0.6	Chard	36	4.0
Tomatoes	123	3.2	Cooked Mustard greens	140	10.3	Plantain	179	0.8	Tangerine	88	0.4	Spinach	25	3.0
Grapefruit	256	2.9	Carrots	110	9.1	Pumpkin bread	60	0.7	Dried Papaya	23	0.2	Kale	25	2.0
Tomato catsup	15	1.8	Parsley	60	3.0	Mandarin oranges, canned	189	0.4	Calamondin	19	0.1	Broccoli	88	1.2
Dried papaya	23	0.7	Kale	25	1.5	Dandelion greens	55	0.2	Kumquat	19	0.04	Cooked Egg yolk	17	0.2

Portion size (Pz); Lycopene (Lyc); β-carotene (β-Car); α-carotene (α-Car); β-cryptoxanthin (β-Cry); Lutein (Lut); Zeaxanthin (Zea)

Carrot, orange, tangerine, tomato, and red pepper have the highest content of carotenoids and all of them have carotenoids that are also called provitamin A (β-carotene, β-cryptoxanthin, and α-carotene) and are typified by the occurrence of a β-ionone ring in the final position of the polyene chain (Toti et al., 2018). Introduced into the body through the diet, provitamin A is transformed into vitamin A in the liver by the action of the enzyme carotenase. Among the functions of vitamin A is the regulation of cell growth and differentiation, but its most important roles are in immune function, eye development and vision (Dias et al., 2018).

The health benefits of carotenoids are based on their antioxidant, immunomodulatory, and anti-inflammatory activities. Carotenoids are also attributed a protective effect against certain types of cancer because they limit abnormal cell growth (Tanaka et al.,

2012). Lutein accumulates in the cerebral cortices and membranes; therefore, it has a protective effect on cognitive abilities in older adults and also has benefits in relation to age-related macular degeneration since the content of lutein and zeaxanthin in the retina increases with the consumption of foods such as spinach, cabbage, and egg yolk (Estévez-Santiago et al., 2016). Finally, other attributions to the healthy properties of carotenoid compounds are their prevention effect of skin cancer by protecting against ultraviolet light. They also contribute to maternal and child nutrition and to genomic effects on transcription/translation (Figure 18) (de Souza Mesquita et al., 2021, Rodríguez-Mena et al., 2022).

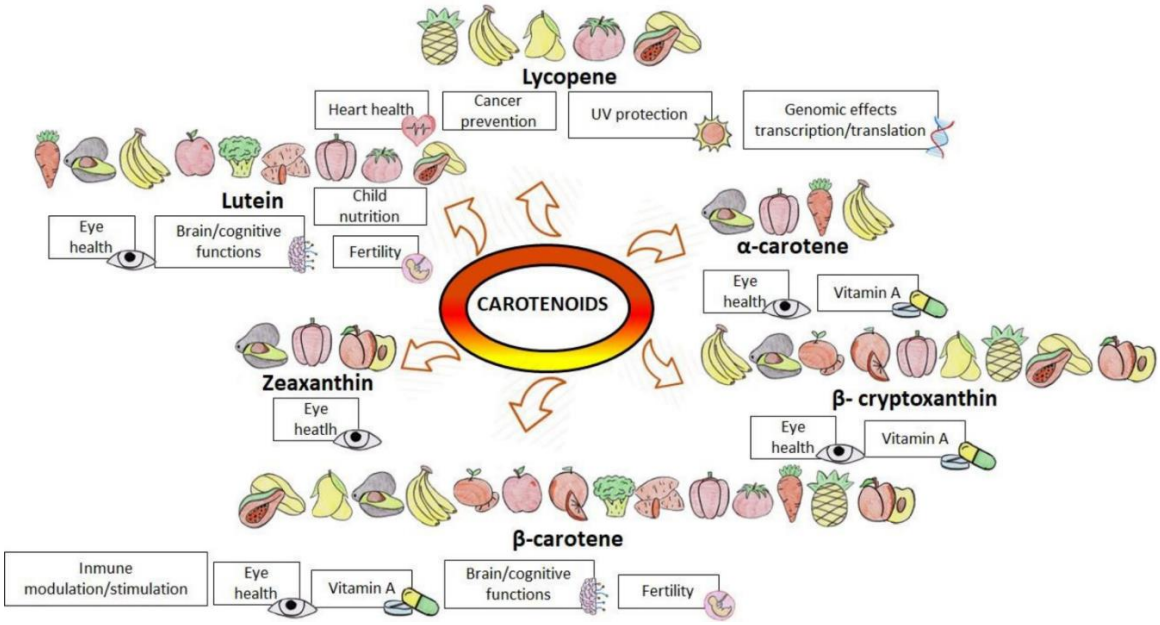


Figure 18. *Carotenoids in fruits and vegetables and their main benefits in human health (From (Rodríguez-Mena et al., 2022)).*

The α- and β-carotene are principally considered as a precursor of vitamin A. Commonly coloured fruits and vegetables such as carrot, sweet potato, apricot, pumpkin and beans are rich sources of these carotenoids. β-carotene has higher conversion efficacy to vitamin A (retinol) then β-Cryptoxanthin and α-carotene (Tang, 2014). In addition to

their pharmaceutical role as vitamin A precursor, these pigments effectively inhibit the number of chronic diseases such as breast and skin cancer. β-carotene has been evaluated for its potential to minimize the risk of lung cancer. However, other *in vitro* studies suggested that multiplication of cancer cells was effectively suppressed by α-carotene than β-carotene (Gallicchio et al., 2008). Additionally, it has also been reported that these pigments activate the cell communication and immune system (Singh et al., 2015).

β-cryptoxanxanthin is also considered as a provitamin A carotenoid and found extensively in citrus fruits, peach, papaya and mangoes. Health benefits of these carotenoids have been scarcely investigated due to domination of other provitamin A carotenoids. β-cryptoxanxanthin has acted as therapeutic agent, especially against bone related diseases. Some studies have shown that β-cryptoxanthin supplementation minimized the chances of osteoporosis by enhancing alkaline phosphatase activity and calcium content in metaphyseal tissue and cortical bone (Yamaguchi, 2008). In addition, this pigment has also upregulate the communication between cells and improve immunity (Burri, 2015, San Millán et al., 2015). Generally, several biological effects of carotenoids have been well documented in published literature, including, anti-inflammatory, antioxidant, immunomodulatory, anticancer and inhibition of mutagenesis (Rao and Rao, 2007).

Lutein and its stereoisomer zeaxanthin are mainly found in calendula flower extract. These are the macular carotenoids that create the macular pigment at the back of the eye. Lutein and zeaxanthin are becoming increasingly important in the nutraceutical market as they are shown to play a significant role in eye health, preventing cataracts and macular degeneration. In humans, lutein and zeaxanthin are thought to function both as antioxidants, thus protecting against oxidative damage, and as filters of ultraviolet light. Lutein is also able to protect the skin from UV-induced damage and can reduce the risk

of cardiovascular disease. Additionally, there is strong epidemiological evidence that lutein may protect against the development of some types of cancer. Additionally, lutein has been shown to enhance the immune response. Generally, these two xanthophylls are not considered toxic and are relatively safe for human consumption. Brain lutein concentrations have been shown to be lower in mild cognitive impairment patients than in those with normal cognitive function (Woodside et al., 2015).

Lycopene is a predominant carotenoid found extensively in fruits and vegetables, with tomato and their products being key sources (Arain et al., 2018). Lycopene can also reduce the risk of cancer, including ovarian, breast, prostate, cervical and liver cancer (Singh et al., 2015). On the other hand, a higher concentration of lycopene effectively reduces the chances of cardiovascular diseases and prevented the oxidative damage of skin due to UV-light damage. In fact, lycopene was marketed as an antioxidant and was proposed for treatment of cardiovascular diseases and prostate cancer. Although there were no differences in the levels of β-carotene, lutein, cryptoxanthin, vitamin E, and vitamin A between the cancer patients, the control levels of lycopene were significantly lower in the cancer patients (Camara et al., 2013).

The consumption of astaxanthin has been associated with a positive role in many human health problems, such as UV-light protection and anti-inflammatory properties, mainly linked to its antioxidant power. Moreover, positive human clinical trials supporting eye health have shown that astaxanthin helps diabetic retinopathy, macular degeneration, eye strain and fatigue, and seeing in fine detail. Moreover, it improves the ability of protective white blood cells to surround and destroy infecting organisms. Astaxanthin also protects human lymphocytes and neutrophils against the oxidant stresses imposed by the actions of certain white blood cells without reducing the killing effects of white blood cells themselves. Epidemiological studies show that increased intake of

carotenoids such as astaxanthin typically lowers risk of many different types of cancer, such as colon and breast cancer. Astaxanthin is best recognized for the pinkish color in aquatic fish and shrimps. It is the strongest antioxidant in carotenoids and thus exhibits stronger antioxidant activity than vitamin E and β-carotene. It has been reported to have the potential to enhance antibody production, antiaging, and sun-proofing, and it also demonstrates anti-inflammatory effects when administered with aspirin (Langi et al., 2018).

1.3.1. Biological Activity of Natural Carotenoids

Many studies have evaluated the safety of carotenoid supplements in cancer patients. These works determined that lycopene supplementation in subjects with prostate cancer was safe and well tolerated. Furthermore, β-carotene is also generally recognized as safe and used as a supplement and nutrient. Hence, carotenoid supplementation is well tolerated by most people (Clark et al., 2006). Carotenoids have demonstrated numerous biological activities and health benefits, for the prevention and treatment of diseases. Figure 19 shows the biological effects and mechanism of action of carotenoid pigments used for the treatment of various diseases.

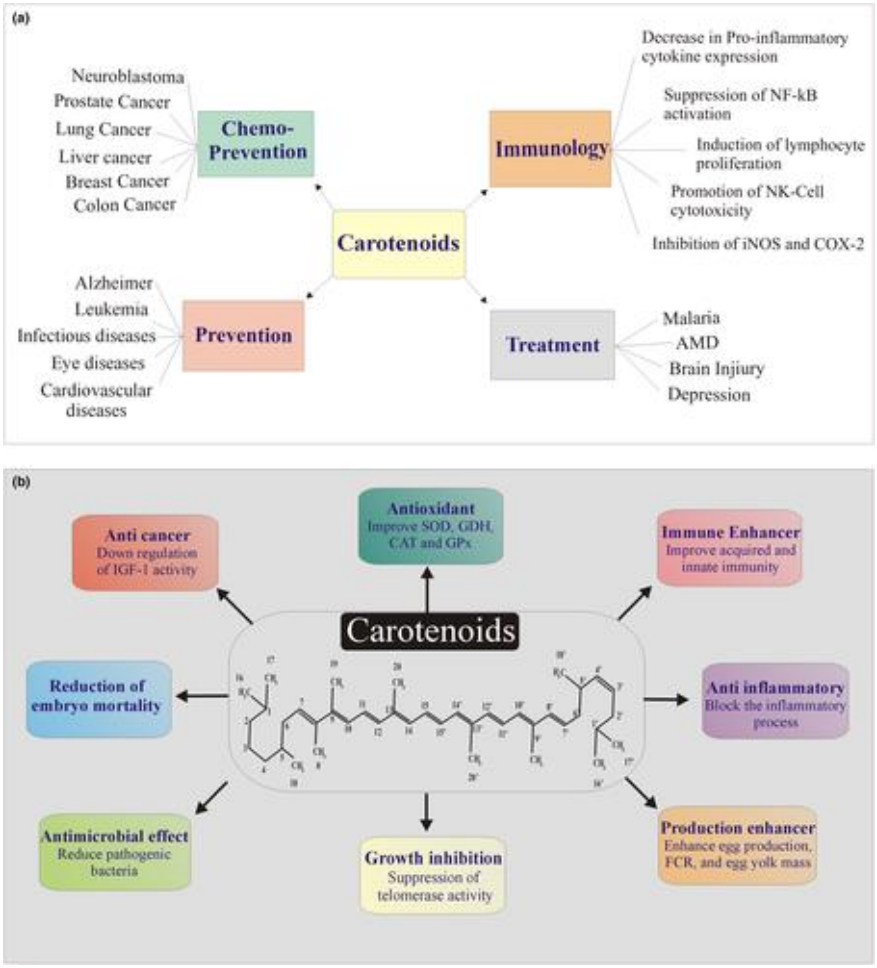


Figure 19. *Biological activities of the carotenoids, (**a**) Showed general effects of the carotenoids on various diseases (**b**) showed molecular mechanism. (From (Nabi et al., 2020)).*

1.3.1.1. Antioxidant Activity

Mainly diseases are caused due to free moving oxygen radicles in the body. Diseases like cancer, cardiovascular diseases, ophthalmic diseases, neurodegenerative diseases are caused due to free radicles. Carotenoids are suggested to protect the body against several ROS-mediated conditions, such as neurological, cancer, eye related and photosensitive disorders. Moreover, certain carotenoids are found to activate the antioxidant gene expression through Nrf2 (Nuclear factor erythroid 2 related factor 2) transcriptional factor which helps in decreasing neurological disorder and diabetes (Aziz et al., 2020).

Ketocarotenoid astaxanthin derived from microalgae mitigates the wide range of oxidative stress via several mechanisms including: downregulation of the production of inflammatory cytokines by decreasing the activation nuclear factor-kB, inhibition of the activity of the renin–angiotensin system, suppression of the production of transforming growth factor b1 and antimicrobial effects (Yuan et al., 2011). The study demonstrated that lycopene is the strongest free radical quencher among 600 natural carotenoids, due to conjugated double bonds and elongated structure. Lycopene also enhances the production of antioxidant enzymes, for example, superoxide dismutase (SOD), glutathione transferase (GST) and quinone reductase through the activation ARE pathways (Sahin et al., 2013).

Among the various defense strategies, carotenoids are most likely involved in the scavenging of two of the reactive oxygen species, singlet molecular oxygen ($^1O_2$), and peroxyl radicals (Young and Lowe, 2001). Singlet oxygen quenching by carotenoids occurs via physical or chemical quenching. Physical quenching involves the transfer of excitation energy from $^1O_2$ to the carotenoid, resulting in ground state oxygen and an excited triplet state carotenoid. The energy is dissipated between the excited carotenoid and the surrounding solvent to yield the ground state carotenoid and thermal energy. In the process of physical quenching the carotenoid remains intact and can undergo further cycles of singlet oxygen quenching. β-Carotene and other carotenoids are the most efficient natural $^1O_2$-quenchers; their quenching activity is closely related to the number of conjugated double bonds present in the molecule. Carotenoids efficiently scavenge peroxyl radicals, especially at low oxygen tension, and contribute to the defense against lipid peroxidation. Under specific conditions carotenoids may also act as prooxidants. Such properties have been determined in vitro and discussed in context with adverse effects observed upon β-carotene supplementation at high levels (Stahl and Sies, 2005).

1.3.1.2. Anti-inflammatory Activity

Inflammation is a biological response of tissue against number of harmful stimuli, such as cell damage, pathogen or irritant (Ferrero-Miliani et al., 2007). The defensive response of body involving activation of immune cells, molecular mediators and blood vessels eliminate the cause of inflammatory process. Several health organizations recommended consistent intake of fruits and vegetables to decrease the incidence of numerous chronic diseases. Phytochemicals such as lipophilic carotenoids suggested that they have excellent anti-inflammatory. Most of these properties have been demonstrated to inhibit or block the inflammatory processes and also block the oxidative stress. Carotenoid supplementation (lycopene, lutein and β-carotene) in the diet of infant decreases the inflammatory response and raise plasma concentration of carotenoids (Nabi et al., 2020). Carotenoids having oxygen in structure like fucoxanthin and astaxanthin has proved to suppress the expression of cytokines IL-6, TNF-α and IL-1β and act as pro and anti-inflammatory compounds. The mechanism behind this action provides that as carotenoids scavenges the oxygen radicle, it will not further able to interact with NF-κB which results into macrophage foam cells and decrease in TNF-α (Bhatt and Patel, 2020).

1.3.1.3. Anticancer Activity

Several experiments show the potential of carotenoids as anti-cancer agents. The major mechanisms through which carotenoids have been implicated in cancer are related to pathways involving cell growth and death. It is seen in most of cases that carotenoids arrest the cell cycle which is associated with down regulation of cyclin D_1, cyclin D_2, CDK4 and CDK6 expression. Consequently, it also up regulates GADD45 α, which inhibits the entry of cell into S phase (Almeida et al., 2020). Moreover, compounds like crocin and crocetin extracted from saffron showed the anti-metastasis properties like anti-

migration, antiinvasive and anti non adhesive effects in combination on 4T1 cell line in breast cancer (Arzi et al., 2020). Carotenoids like β-cryptoxanthin and lycopene are found suppressing the NF-κB signalling pathway which is effective against lung cancer and prostate cancer (Lim and Wang, 2020). β-carotene has found to have anti angiogenic activity that is it helps to halt the process of developing new blood vessels which is often seen in cancerous tumours. Lycopene induces both cell cycle arrest and apoptosis. Growth factors, such as PDGF and IGF, enhance cell survival by protecting cells from apoptosis. Lycopene blocks cell-cycle progression from G1 to S phase. Furthermore, increases the levels of tumor suppressor p53. Lycopene promotes apoptosis by decreasing Bcl-2 and survivin expression, increasing the levels of the proapoptotic proteins Bax, Bad, Bim, and Fas ligand, and activating caspases 8, 9, and 3 (Figure 20) (Bodade and Bodade, 2020, Trejo-Solís et al., 2013).

Lycopene and its metabolites, through their strong antioxidant capacity, block the "initiation" phase by neutralizing ROS, by activating detoxification systems and the expression of antioxidant enzymes, thus protecting cells from damage caused by carcinogenic substances. Lycopene can also block or prevent tumor promotion and progression by modulating inflammatory cytokine and growth factor pathways (Figure 20).

A previous study proposed that the effects of lycopene may be attributed to the induction of antioxidant enzymes and phase II detoxifying enzymes. Nrf2 is an important transcription factor that plays a role in the detoxification of carcinogens agents and anti-oxidant cellular defense systems. Under physiological conditions, Nrf2 is sequestered in the cytoplasm by inhibitory protein Keap1 (kelch-like ECH-associated protein 1), a cytoskeletal protein which in turn serves as a substrate for Cul3, a marker of the ubiquitination system. Cul3, by continuously tagging Nrf2 with ubiquitin, accelerates its

26S proteasome degradation, preventing the overexpression of target genes controlled by the Nrf2/Keap1 system. However, in case of oxidative stress, the electrophilic species release generates the complexes formation between sulfhydryl groups of the Keap1 cysteine residues. This causes the detachment of the Keap1 protein from Nrf2, that translocated into the nucleus, resulting in the up regulation of cytoprotective gene sequences and recruiting co-activators of the Maf proteins family (Figure 20) (Trejo-Solís et al., 2013, Marino et al., 2023).

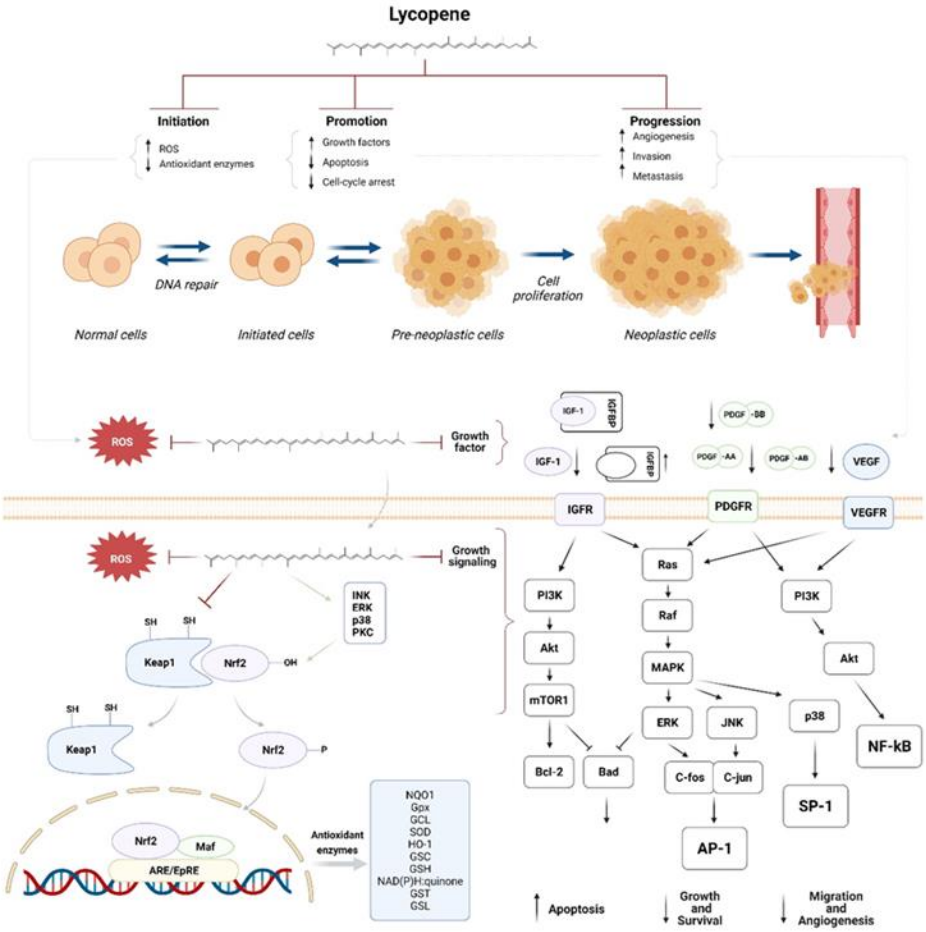


Figure 20. *(**Top**) Phases of lycopene intervention in the carcinogenic process. (**Bottom left**) A possible mechanism of Nrf2 regulation by lycopene. (**Bottom right**) Lycopene induces cell-cycle arrest and apoptosis.*

1.3.1.4. Cardiovascular Diseases (CVD)

Natural carotenoids derived from plants and vegetables, mainly lycopene, α-carotene, β-carotene, zeaxanthin, lutein and β-cryptoxanthin efficiently reduce the risk of cardiovascular disease. Supplementation of β-carotene (50 mg/day) minimizes the mortality due to cardiovascular disease, while β-carotene supplementation with or without aspirin significantly reduces the risk of myocardial infarction. Nevertheless, also other carotenoids such as lutein, β-cryptoxanthin, astaxanthin should be considered to potentially reduce the chances of cardiovascular disorders (Nabi et al., 2020).

Certain carotenoids like astaxanthin, lutein and β cryptoxanthin are found more involved in preventing cardiovascular disease by oxidizing LDL and decreasing HDL. This can be used in treating myocardial injury and many more. Hence, it can be used as food additives to reduce the obesity associated cardiac dysfunction (Pashkow et al., 2008).

1.3.1.5. Skin Protection Activity

β-carotene supplements are widely used as so-called oral sunscreens and the protective effects are thought to be related to the antioxidant properties of the carotenoid (Sies and Stahl, 2004). Upon UV-irradiation, the skin is exposed to photooxidative damage which is induced by the formation of reactive oxygen species. Photooxidative damage affects cellular lipids, proteins and DNA and is considered to be involved in the pathobiochemistry of erythema formation, premature aging of the skin, development of photodermatoses, and skin cancer. Several studies in humans have shown that carotenoid levels in plasma and skin decrease upon UV-irradiation; lycopene is lost preferentially as compared to other carotenoids. Therefore, numerous studies have demonstrated the beneficial effects of supplementation of single carotenoids, β-carotene, lutein and lycopene, and a mixture of the same in protecting or improving UV-induced erythema in

humans. Protection against UV-induced erythema was also observed after the dietary intervention. Tomato paste contains high amounts of the tomato-specific carotenoid lycopene and was selected as a natural dietary source providing carotenoids to protect against UV-induced erythema in humans. The ingestion of tomato paste led to elevated serum levels of lycopene and an increase of total carotenoids in skin. After treatment, erythema formation was significantly reduced, demonstrating that UV-induced erythema can be ameliorated by dietary intervention (Stahl and Sies, 2005).

1.3.1.6. Neurodegenerative Diseases

In nervous system increase of oxidative stress results into several neurodegenerative diseases such as Alzheimer's, Huntington's, Parkinson's and amyotrophic lateral sclerosis (ALS). Several diseases are due to Ca^{2+} inability to signal the molecules but carotenoids like astaxanthin, βcarotene and lycopene are involved in Ca^{2+} ion transportation in brain, with proper dietary of carotenoids malfunction due to improper signalling can be reduced. The ability to cross the blood brain barrier, and cell mitochondrial membrane with stability along with antioxidant property carotenoid-astaxanthin can be able to reduce the risk of diseases related to the nervous system (neurodegenerative). In addition to its Astaxanthin can combat neurodegenerative diseases by different properties such as anti-apoptosis, reduction in cerebral infarction in brain tissue, lowers ischemia by induced apoptosis, reduction of glutamate release and reduce free radical damage (Rzajew et al., 2020, Kowsalya et al., 2019). Moreover, serum lutein, zeaxanthin and β-carotene were most consistently related to better cognition, whilst brain levels of lutein and β-carotene were also related to cognition. Brain lutein concentrations have been shown to be lower in mild cognitive impairment patients than

in those with normal cognitive function, whilst, lutein supplementation improved verbal fluency, memory and rate of learning (Johnson et al., 2013, Johnson, 2012).

1.3.2. Bioavailability

Bioavailability refers to the portion of the carotenoid which is absorbed in the body, enters in systemic circulation and becomes available for utilization in normal physiological functions or for storage in the human body. Due to their largely hydrocarbon structure, carotenoids tend to be non-polar and need dietary fat to be absorbed into the intestinal lumen (Cervantes-Paz et al., 2017). Once released from their food matrix, carotenoids are incorporated into the lipid phase and then emulsified in small lipid droplets in the stomach. From the lipid droplets, the carotenoids are incorporated into micelles formed by bile salts, bile phospholipids, dietary lipids and their hydrolysis products action. The micelles subsequently migrate to the intestinal epithelium where the carotenoids are absorbed by the enterocytes. Intestinal absorption of carotenoids occurs mainly through a passive diffusion mechanism. However, some studies suggest the existence of an active transport mediated by the SR-BI receptor (Scavenger Receptor class B type I). Once absorbed, the carotenoids are packaged in chylomicrons, secreted in-to the lymphatic system and transported to the liver (Figure 21) (Shardell et al., 2011, Moussa et al., 2008).

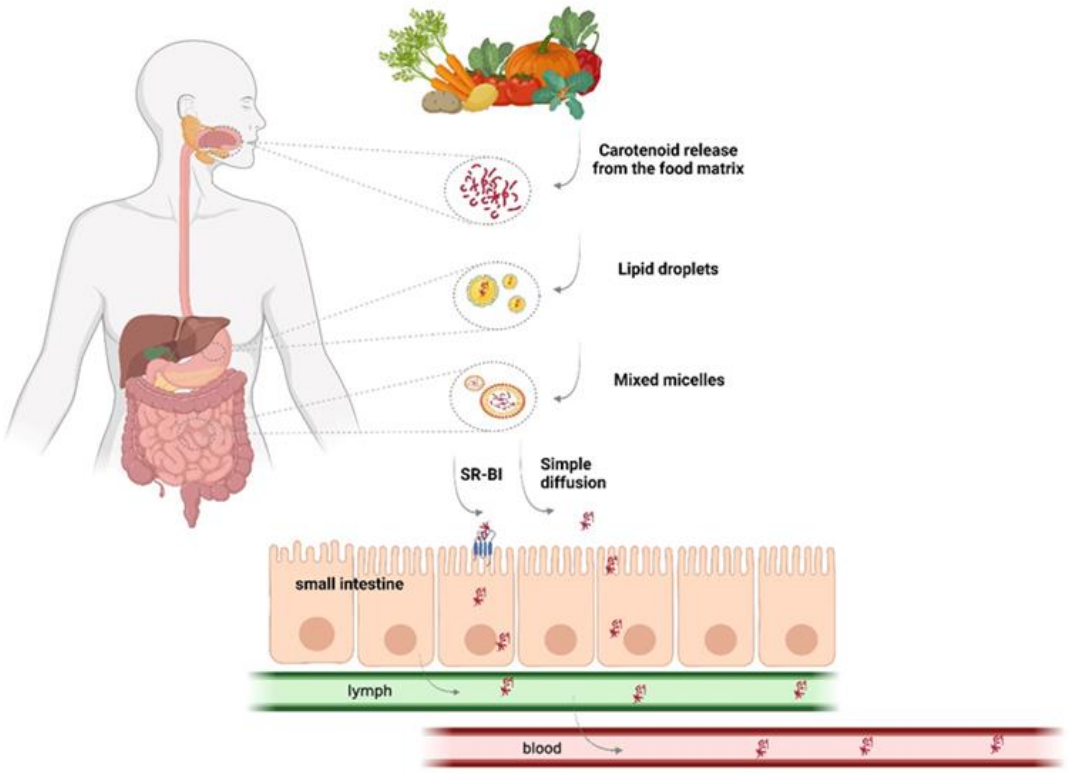


Figure 21. *Schematic representation of carotenoids absorption.*

The bioavailability of β-carotene from plant sources is generally low (10–65%), due to resistance of carotene–protein complexes, fibers and the plant cell walls to digestion and degradation to achieve adequate release of carotenoids (Rein et al., 2013). Thus, soluble proteins have been shown to inhibit the incorporation of β-carotene into the gastric emulsion and bile salts, indicating that the interfacial characteristics of the gastric emulsion determine the extent of carotenoid absorption in intestine. The food matrix plays a significant role on bioavailability because release from the food matrix is a primary factor limiting bioavailability of carotenoids. Release of carotenoids depends on the level of digestion and degradation of the food matrix, which may be assisted by mechanical processing prior to digestion. Mechanical processing helps in reducing particle size, results in greater surface area to come into contact with pancreatic lipases and bile salts to improve digestion and release. Absorption of carotenoids occurs only

when they are mixed with micelles, so factors affecting the micelle formation, also affect the bioavailability of carotenoids. Since lipids are required for incorporation into micelles and also stimulate release of bile to facilitate micelle formation, addition of dietary fat improves the bioavailability of carotenoids (Lemmens et al., 2014).

CHAPTER II:

Objectives of the Research

2. Objectives of the Research Project

The aim of the research project is the isolation and characterization of compounds with potential antiproliferative activity starting from natural and food matrices coming from the Mediterranean area, in order to use them as adjuvants in cancer therapies or as a prevention treatment. The purpose is to treat food not only in the traditional way in which it provides food, energy and various components necessary to meet the basic nutritional needs of every human being, but to identify the foods that have properties in the treatment of chronic diseases. The project will allow to identify new active molecules with potential anticancer properties to be used in addition to existing treatments. In particular, the objectives of project are:

- Identification and study of different matrices containing molecules with onconutraceutical potential properties;
- Development and implementation of an experimental protocol suitable for the extraction of bioactive compounds from natural matrices;
- Development and optimization of highly efficient analytical platforms for the identification of the main bioactives isolated from complex matrices;
- *In vitro* study of the biological properties of bioactive molecules on different cell lines.

In the first and second year, my research project had as its main objective the evaluation of the onconutraceutical potential of two food matrices of plant origin such as *Citrus sinensis* and *Humulus lupulus*. Subsequently during the third year I concentrated on the study of further matrices focusing more on *Lycium barbarum*.

CHAPTER III:

Mediterranean Area: *Citrus sinensis* and *Humulus lupulus*

3. Evaluation of Biological Properties of Fractions Extracts from *Citrus sinensis* and *Humulus lupulus*

3.1. Citrus sinensis

Citrus is the largest genus in family *Rutaceae* with ca. 70 species to include various edible species, such as *C. limon* (lemon), *C. medica* (citron), *C. aurantium* (sour orange), *C. paradisi* (grapefruit), *C. reticulata* (mandarin, tangerine), *C. clementina* (clementine), *C. bergamia* (Bergamot), *C. junos* (Yuzu), *C. japonica* (Kumquat), and *C. sinensis* (sweet orange) (Farag et al., 2020). *Citrus* species are native Asian crops from which they have spread to over 140 countries worldwide (Bora et al., 2020). With more than an annual production of 124.3 million tons worldwide, *Citrus* plants represent the largest crops cultivated worldwide (Mahato et al., 2020). As important examples, sweet and bitter oranges are the most cultivated worldwide among *Citrus* trees with a total production of ca. 75 million tons based on FAOSTAT, 2020 reports (Farag et al., 2020).

C. sinensis (L. Osbeck) or sweet oranges (Figure 22), represents the largest citrus cultivar groups grown around the world, accounting for about 70% of the total annual production of *Citrus* species (Favela-Hernández et al., 2016). *C. sinensis* is consumed all over the world as an excellent source of vitamin C, which is a powerful natural antioxidant that builds the body's immune system (Etebu and Nwauzoma, 2014). It has been used traditionally to treat ailments like constipation, cramps, colic, diarrhea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress (Milind and Dev, 2012). Also, more recently it has been reported for use in the management of patients with arthritis, asthma, Alzheimer's disease,

Parkinson's disease, macular degeneration, gallstones, diabetes mellitus, multiple sclerosis, cholera, gingivitis, cataracts, ulcerative colitis, Crohn's disease (Braidy et al., 2017).

Figure 22. *Citrus sinensis fruits.*

C. sinensis contains numerous secondary metabolites that contribute to the various pharmacological activities attributed to this plant. Several phytoconstituents have been identified in the fruit, peel, leaves, juice and roots of *C. sinensis*, including: flavonoids, steroids, hydroxyamides, alkanes and fatty acids, coumarins, peptides, carbohydrates, carbamates and alkylamines, carotenoids, volatile compounds, and nutritional elements such as potassium, magnesium, calcium and sodium The results of numerous studies demonstrate that orange is a good source of bioactive compounds that could be used in products as functional ingredients and for the realization of new drugs (Favela-Hernández et al., 2016).

The most important bioactive compounds of *C. sinensis* are flavonoids. Generally, citrus flavonoids are composed of three major subgroups including flavanones, flavonoid glycosides, and polymethoxyflavones (PMF). In particular, polymethoxyflavones are methoxylated bioflavonoids present almost exclusively in the genus *Citrus*, in particular in the peel of sweet oranges (*Citrus sinensis*) and mandarins (*Citrus reticulata*). PMFs are chemically flavones characterized by the presence of two or more methoxy groups on

their benzo-γ-pyrone base skeleton (15-carbons, C6-C3-C6) with a carbonyl group in the C4 position (Gao et al., 2018).

So far, more than 20 polymethoxylated flavonoids have been isolated and identified from different citrus plants. In particular, Nobiletin and Tangeretin are the most abundant PMFs in citrus peel (Figure 23) (Tung et al., 2019).

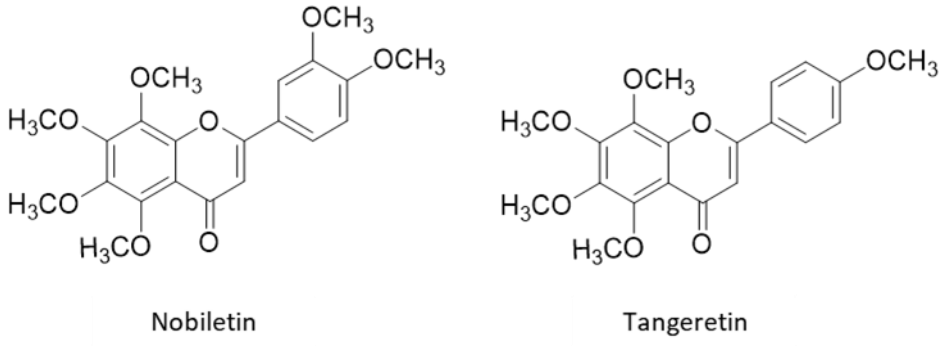


Figure 23. *Structures of Nobiletin and Tangeretin.*

PMFs have been shown to block adhesion molecule biosynthesis by cytokine-induced endothelial cells, to block activation-induced degranulation of neutrophils and mast cells, to inhibit expression of tumor necrosis factor-α (TNFα), to reduce the invasiveness of tumors in animal models, to induce the differentiation of myeloid leukemic cells, to suppress proliferation while promoting apoptosis, to reduce lymphocyte proliferation and platelet aggregation. Because of the hydrophobic nature of methoxy groups as compared to hydroxyl groups, PMFs are more lipophilic as compared to polyhydroxylated flavonoids, such as quercetin, luteolin, and narigenin. Therefore, the PMFs may have higher permeability through the small intestine and are readily absorbed into the blood circulation system of the human body (Li et al., 2006). Furthermore, PMFs present in the *citrus* peel are able to reduce blood cholesterol levels, with a mechanism of action equal

to that of statins and with greater efficacy than some commonly used drugs, without showing any side effects (Milind and Dev, 2012).

The pharmacological properties of *Citrus sinensis* have been extensively studied and among these the antioxidant, anti-inflammatory, cardioprotective, antiulcerative, antibacterial and antitumor properties are particularly interesting (Etebu and Nwauzoma, 2014).

Numerous studies indicate that orange flavonoids have important antioxidant properties, they are excellent *radical scavengers*, thanks to their ability to inhibit the hydroxyl radical (·OH) and donate hydrogen atoms (Senanayake et al., 2005). Many *in vitro* and *in vivo* studies indicate protective effects of polymethoxyflavones against the onset of cancer. PMFs have been reported to inhibit carcinogenesis by mechanisms such as blocking the metastasis cascade, inhibition of tumor cell mobility in circulatory systems, apoptosis, selective cytotoxicity, and antiproliferation (Li et al., 2006).

Most of the studies on the biological activity of *Citrus* in recent years have focused on Tangeretin and Nobiletin, the most abundant PMFs in citrus peels. In particular, Nobiletin can inhibit the proliferation of human prostate, skin, breast and colon cancer cell lines, inhibit the production of some matrix metalloproteinases (MMPs), and inhibit the proliferation and migration of human umbilical endothelial cells (Ashrafizadeh et al., 2020). Nobiletin has also been shown to have antiproliferative and apoptotic effects on a gastric cancer cell line and has a destructive effect on cell cycle progression. In a study of 42 flavonoids, Nobiletin showed the strongest antiproliferative activity against six human cancer cell lines (Yoshimizu et al., 2004).

More recent studies have demonstrated that Nobiletin and its derivatives are able to act at multiple pathways implicated in cancer progression and to inhibit many of the hallmarks of specific colorectal cancer (CRC) pathophysiology, including arrest cycle,

inhibition of cell proliferation, induction of apoptosis, prevention of tumor formation, reduction of inflammatory effects and limitation of angiogenesis (Goh et al., 2019).

3.2. Humulus lupulus

The genus *Humulus*, belonging to the *Cannabaceae* family, consists of three species: *Humulus lupulus* Linneus, *Humulus japonicus* Siebold & Zucc. and *Humulus yunnanensis* Hu. *Humulus lupulus* (hops) is a perennial herbaceous liana, native to China, naturalized in central Europe and widely cultivated in all temperate regions of the world (Korpelainen and Pietiläinen, 2021).

Hops, one of the main raw materials of beer, has been used extensively throughout the world in the brewing industry. It acts as a preservative to give beer its unique aroma and flavor (Dostálek et al., 2017). The female inflorescences (Figure 24), the hop cones (or hops), rich in polyphenolic compounds and acylfluoroglucides play a key role in defining the aroma and bitter taste of beer, but also as a natural antibacterial agent (Moir, 2000). Furthermore, hops has been used as a medicinal plant for a long history due to its richness in a variety of phenolic compounds (Zanoli and Zavatti, 2008). Several phytochemical studies have been conducted focusing on the study of the composition of hop cones and other parts of the plant, which allowed to isolate and identify pharmacologically relevant compounds such as phenolic acids, prenylated chalcones, flavonoids, catechins and proanthocyanidins (Nikolic and B Van Breemen, 2013).

Figure 24. *Humulus lupulus.*

The major structural classes of phytochemical compounds identified from mature hop cones include terpenes, bitter acids, and chalcones. Bitter acids are derivatives of fluoroglucinol generally classified as α-acids and β-acids. Hops are also rich in flavonolic glycosides (kaempferol, quercetin, quercitrin, rutin) and catechins (catechin gallate, epicatechin gallate) (Zanoli and Zavatti, 2008).

A key subclass of flavonoids is that of prenylflavonoids, which combine a flavonoid skeleton with a lipophilic prenyl side chain (Yang et al., 2015). Prenylation usually enhances the bioactivity of flavonoids. The mechanism of action of prenylation is based on the increase in the lipophilicity of flavonoids, which translates into a greater affinity of the compound with biological membranes and a better interaction with target proteins (Xu et al., 2012).

Several prenylflavonoids were identified from hop cones (Figure 25). The most abundant prenylated flavonoid in hops is xanthohumol (XN). In nature, XN exists ubiquitously within hops plant, with a content of 0.1% - 1% (dry weight) in the female inflorescences. (Liu et al., 2015). Numerous studies have revealed a wide variety of bioactivities for prenylflavonoids, especially estrogenic, antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, antiplasmodial, immunomodulatory, and antitumor activity (Jiang et al., 2018). Xanthohumol and other prenylated chalcones have attracted considerable attention in recent years as cancer chemopreventive agents, while 8-

Prenylnaringenin (8-PN), an isomerization product of desmethylxanthumol also found in beer, is considered one of the most potent phytoestrogens currently known (Zanoli and Zavatti, 2008).

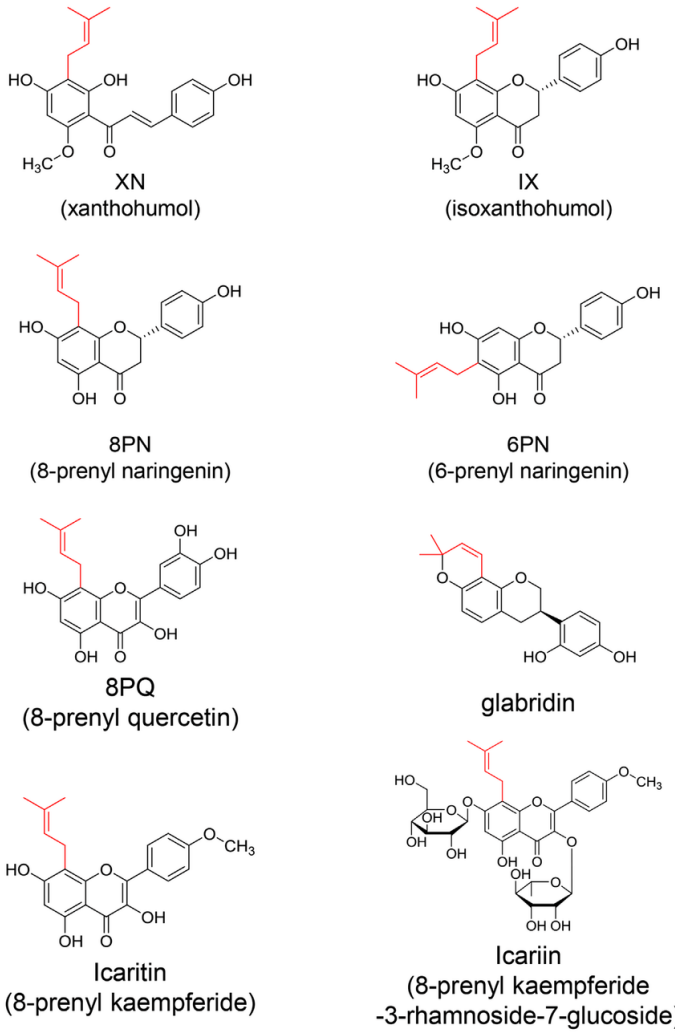


Figure 25. *Chemical structures of prenylflavonoids found in hops.*

In recent years there has been a focus on the estrogenic and chemopreventive properties of hops and some active compounds, such as 8-Prenylnaringenin and Xanthohumol have received much attention. 8-Prenylnaringenin, the most representative nutraceutical, found in hops and beer, showed comparable binding activity with both

estrogen receptor isoforms (ERα and ERβ), suggesting a potential hormonal activity of 8-PN (Yang et al., 2015).

Xanthohumol shows good immunosuppressive effects on T-cell proliferation, IL-2-activated killer cell development, T-lymphocytes, and Th1 cytokine production (Gao et al., 2009). These effects are partly due to inhibition of the nuclear factor transcription factor NF-κB through suppression of IkBα phosphorylation (Yang et al., 2015).

Several *in vitro* and *in vivo* studies have evaluated the potential activity of hop components, in particular xanthohumol, as chemopreventive agents. Numerous evidences suggested the antitumor activity of xanthohumol against non-small cell lung cancer (NSCLC), leukemia, hepatocellular carcinoma (HCC), breast cancer, prostate cancer (PCa), cholangiocarcinoma (CCA), glioblastoma, pancreatic cancer, colon cancer, cervical cancer, melanoma, thyroid cancer, laryngeal squamous cell carcinoma (LSCC), and ovarian cancer (Jiang et al., 2018). Xanthohumol has shown numerous inhibitory mechanisms in the initiation (reactive molecules, DNA damage), promotion (mutation, modified cell structure) and progression (uncontrolled cell growth, tumors, metastasis) phases of carcinogenesis and in particular, the antitumor capacity was highlighted by inhibition of the initiation and development of carcinogenesis, inhibition of proliferation, induction of apoptosis and inhibition of migration and angiogenesis (Liu et al., 2015).

The investigation of the anti-cancer effect of xanthohumol in colon cancer cells was shown that this compound induced G2/M cell cycle arrest and apoptosis by increasing caspases, Bax/Bcl-2 ratio and PARP cleavage (Liu et al., 2019b, Liu et al., 2020a). In addition, the induction of cell cycle arrest in HT-29 cells with XN treatment resulted in the suppression of cyclin B1 and Ras/MEK/ERK pathway (Liu et al., 2019b). A recent study has shown that, the treatment with xanthohumol inhibited glycolysis and cell proliferation through the reduction of hexokinase-2 (Hk-2), enzyme overexpressed in

colon cancer and that the suppression may inhibit survival and proliferation (Liu et al., 2019a). Xanthohumol has also been reported to sensitize CRC cells to chemotherapy (Scagliarini et al., 2020).

Studies show that in CRC cell lines, the polymethoxyflavone Nobiletin inhibits proliferation, induces cell apoptosis, limits angiogenesis, sensitizes cells to chemotherapy and prevents tumor formation. As well as, the prenylflavonoid Xanthohumol is capable of inhibiting cell proliferation, inducing DNA damage and apoptosis, and sensitizing CRC cell lines to chemotherapy. For these reasons, I carried out a study on the anticancer potential of extracts of *Citrus sinensis* and *Humulus lupulus* with the aim of verifying their nutraceutical value which could offer health benefits.

3.3. Materials and Methods

3.3.1. Simple Preparation

Citrus sinensis var. Tarocco, supplied by the company "Agrumaria Corleone" (Palermo, Italy), was squeezed by hand and subsequently centrifuged at 15000 × g for 15 minutes at 25°C to remove fibers, then freeze-dried for 24 hours at -52°C (LyoQuest-55, Telstar Technologies, Terrassa, Spain). The resulting powder was extracted with MeOH (Sigma-Aldrich) and the procedure was repeated three times for the complete recovery of the polyphenolic fraction (Pepe et al., 2017).

Hop inflorescences were converted to powder with a mortar and treated first with hexane for 10 min and then extracted with MeOH for 10 min (×3) (Salviati et al., 2019). The methanolic extracts were combined, evaporated to dryness under vacuum at 40°C in a rotary evaporator, dissolved in MeOH:water 50:50 (v/v) at a concentration of 1 mg/mL, filtered on a 0.45 µm nylon membrane (Merck Millipore, Milan, Italy), and finally analyzed by reversed-phase liquid chromatography (RP)-ultra-high performance

(UHPLC) coupled to diode array detection (DAD) and mass/mass spectrometry (MS/MS).

3.3.2. LCMS–IT-TOF Analysis of Citrus sinensis and Humulus lupulus Extracts

UHPLC-ESI-IT-TOF analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 AR5 degasser, a CTO-20A column oven, an SPD-M20A photo diode array detector (equipped with a 2.5 μL detector flow cell volume), a SIL-30AC autosampler. The UHPLC system was coupled online to an LCMS–IT-TOF mass spectrometer through an ESI source (Shimadzu, Kyoto, Japan). LC-MS data elaboration was performed by the LCMSsolution® software (Version 3.50.346, Shimadzu). LC-MS analysis of polyphenolic compounds was carried out on Kinetex™ EVO C18 150×2.1 mm (100 Å), packed with 2.6 μm particles, column (Phenomenex, Bologna, Italy). Flow rate was 0.5 mL/min. Column oven temperature was set to 40°C. Injection volume was 2 μL. Chromatograms were monitored at the maximum wavelength of the biomolecules of interest, i.e., at 330 nm for the bioactive compounds isolated from the orange and at 370 nm for the polyphenols extracted from hops. UHPLC system was coupled on-line to a hybrid IT-TOF instrument, flow rate from LC was splitted 50:50 prior of the ESI source by means of a stainless steel Tee union (1/16 in., 0.15 mm bore, Valco HX, Texas U.S). Resolution, sensitivity, and mass number calibration of the ion trap and the TOF analyzer were tuned using a standard sample solution of sodium trifluoroacetate. After the calibrant had flowed, cleaning operation of the tube and ESI probe was made by flowing acetonitrile (0.2 mL/min, 20 min).

Mobile phase for the analysis of *Citrus* consisted of 0.1% (v/v) HCOOH/H_2O (A) and 0.1% (v/v) HCOOH/ACN (B). Analysis was performed in gradient elution as follows: 0-

2.50 min, 5-15% B; 2.50-10.00 min, 15-25% B; 10-12.00 min, 25-55% B; 12-14.50 min, 55-65% B; 14.50-17.00 min, 65-70% B. MS detection of polyphenol extracted from *Citrus sinensis* was operated both positive and negative ionization mode with the following parameters: detector voltage, 1.55 kV; CDL (curve desolvation line) temperature, 200°C; block heater temperature, 200°C; nebulizing gas flow (N2), 1.5 L/min; drying gas pressure, 100 kPa. Full scan MS data were acquired in the range of 200-800 m/z (ion accumulation time, 40 ms; IT, repeat=2). MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 150-800 m/z; peak width, 3 Da; ion accumulation time, 60 ms; CID energy, 50%; collision gas, 50%; repeat =1; execution trigger (BPC) intensity, at 95% stop level.

LC-MS analysis of *Humulus lupulus* extract was carried out employing as mobile phases: A) 0.1 % CH_3COOH in H_2O, B) ACN plus 0.1 % CH_3COOH with the following gradient: 0-15.00 min, 5-30% B; 15-20.00 min, 30-70% B; 20-22.00 min, 70-95% B; 22-25.00 min, isocratic to 95% B; 25-30.00 min, 5% B. MS analysis was operated in negative ionization mode with the following parameters: detector voltage, 1.53 kV; CDL temperature, 250°C; block heater temperature, 250°C. Full scan MS data were acquired in the range of 150-2000 m/z. MS/MS experiments were conducted in data dependent acquisition, precursor ions were acquired in the range 150-900 m/z; peak width, 3 Da; ion accumulation time, 45 ms; CID energy, 50%, collision gas 50%.

Identification of bioactive compounds was accomplished by comparing the retention times, UV-VIS spectra, mass spectra, and MS/MS fragmentation patterns of isolated analytes with those of commercially available standards. The data obtained were finally compared with those reported in the literature. The molecular formulas of the identified compounds were obtained using the Formula Predictor software (version 1.12.0, Shimadzu), setting a low tolerance value so that most of the identified compounds were

in position 1 in the list of possible candidates. The following online databases were also consulted: ChemSpider (http://www.chemspider.com), SciFinder Scholar (https://scifinder.cas.org) and Phenol-Explorer (www.phenol-explorer.eu).

3.3.3. Semiprep-RPHPLC-UV/Vis

The polyphenolic extracts from *Citrus sinensis* and *Humulus lupulus* were fractionated by semi-preparative reversed phase liquid chromatography. For the separation a Shimadzu Semiprep-HPLC was employed consisting of two LC 20 AP pumps, a SIL 20 AP autosampler, a fraction collector FRC 10A, a UV detector SPD 20 A equipped with a preparative cell and a system controller CBM 20 A.

The separation was carried out on a Kinetex^{TM} C18 150 × 21.2 mm × 5 µm (100 Å), flow rate 20 mL/min, injection volume 5 mL (2 mg/mL). The optimal mobile phase consisted of (A) H_2O and (B) ACN both acidified by formic acid and acetic acid 0.1% (v/v) for polyphenol Citrus and Hops analysis, respectively. Analysis was performed in gradient elution as follows:

Citrus sinensis gradient, 0-30.00 min, 10-70 % B; 30-35.00 min, 70-10% B; 35-40.00 min, isocratic to 10% B.

Humulus lupulus gradient, 0-15.00 min, 5-30 % B; 15-20.00 min, 30-70% B; 20-22.00 min, 70-100% B; 22-27.00 min, isocratic to 100%; then five minutes for column re-equilibration.

3.3.4. Cell Culture

The purification and culture of CSphCs, from 6 primary tumor specimens and 6 liver metastasis of patients diagnosed with CRC, were performed as described in (Todaro et al., 2007), in accordance with the ethical standards of Human Experimentation. The

authentication of CRC sphere cells (CR-CSphCs) is routinely performed by the short tandem repeat (STR) DNA profiling kit (GlobalFiler™ STR kit, Applied Biosystem, Thermo Fisher Scientific, Waltham, MA, USA) followed by sequencing analysis on ABIPRISM 3130 (Applied Biosystem, Thermo Fisher Scientific Waltham, MA, USA). Mycoplasma infection is constantly monitored with the MycoAlert TM Plus Mycoplasma Detection Kit (Lonza, Houston, TX, USA). DNA profiles of patient tumor tissues were matched with the corresponding CR-CSphCs.

HCT116 and RKO CRC cell lines were purchased by ATCC (Manassas, VA, USA) and cultured in DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Corning, Corning, NY, USA). HUVEC and HS-5 cell lines were purchased by ATCC (Manassas, VA, USA) and cultured in the Vascular Cell Basal Medium supplemented with the Vascular Endothelial Cell Growth Kit-VEGF (ATCC, Manassas, VA, USA) and in DMEM (ATCC, Manassas, VA, USA) supplemented with 10% FBS, respectively.

CRC cells were treated with 5-fluorouracil (Selleckchem, Houston, TX, USA) plus oxaliplatin (Sigma-Aldrich, St. Louis, MO, USA). Oxaliplatin was administered 3 h before 5-fluorouracil.

3.3.5. Cell Viability Assay

Cell viability was determined by adding the CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI, USA) to untreated and treated CRC stem cells (CR-CSCs) and CRC cell lines. The solution was incubated for 2 h at 37 °C and the 490 nm absorbance was assessed by using the Programmable MPT plate reader (GVD).

Cell viability was assessed with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in the two normal cell lines, HUVEC and HS5. Twenty-

four hours after seeding, the cells were treated with 5–10 and 25 µg/mL of orange fractionated extract, hop fractionated extract, and mix extract. After 24 or 48 h treatment, MTT was added to each well and the plate was incubated for 3 h at 37 °C. After the addition of isopropanol, the plate was read at 540 nm.

3.3.6. Drug Combination Study

Drug combination studies have been assessed by using the Chou–Talalay method, which is based on the median effect and the combination index (CI) equations in order to determine the quantization of drug interactions. Synergy plots are based on the CI, computed in CompuSyn using the Chou–Talalay method, calculated on cell proliferation following the treatment with different FOX and Nobiletin dose pairs. CI < 1 represented synergism (slight, moderate, strong, very strong); otherwise, it indicated additivity (CI = 1) or antagonism (CI > 1) between two drugs (Chou, 2006).

3.3.7. RNA Isolation and Gene Expression Analysis

The purification of RNA was carried out using TRIZOL (Thermo Fisher Scientific, Waltham, MA, USA) protocol. For gene expression analysis, the total RNA (1 µg) was retrotranscribed and subjected to quantitative real-time PCR (qRT-PCR) with a custom PrimePCR panel (Bio-Rad, Hercules, CA, USA) for 88 genes involved in cell death, stemness, and the epithelial-to-mesenchymal transition according to the manufacturer's instructions. Single gene assays were also performed using an SYBR green PCR mastermix (Qiagen, Hilden, Germany) and the following primers: *DKK1* (forward: 5′- GGT ATT CCA GAA GAA CCA CCT TG -3′; reverse: 5′- CTT GGA CCA GAA GTG TCT AGC AC -3′); *WNT5B* (forward: 5′- CAA GGA ATG CCA GCA CCA GTT C -3′; reverse: 5′- CGG CTG ATG GCG TTG ACC ACG -3′); *WNT3A* (forward: 5′- ATG AAC

CGC CAC AAC AAC GAG G -3′; reverse: 5′- GTC CTT GAG GAA GTC ACC GAT G -3′); *WNT7B* (forward 5′- AGA AGA CCG TCT TCG GGC AAG A -3′; reverse 5′- AGT TGC TCA GGT TCC CTT GGC T -3′). The mRNA level was normalized to *GAPDH* (forward: 5′- GCT TCG CTC TCT GTC CCT CCT GT -3′; reverse: 5′- TAC GAC CAA ATC CGT TGA CTC CG -3′) housekeeping gene and calculated using the CT comparative method (ΔΔCt method).

3.3.8. Flow Cytometry

CR-CSCs were washed in PBS twice, and stained for 1 h at 4 °C with conjugated antibodies CD44v6-APC (2F10, mouse IgG1, R&D systems, Minneapolis, MN, USA) or isotype-matched control (IC002A, mouseIgG1, R&D systems, Minneapolis, MN, USA). Dead cells were excluded based on the uptake of 7-AAD (BD Biosciences, Franklin Lakes, NJ, USA).

For cell cycle analysis, untreated and treated CR-CSC were washed with PBS and centrifuged at 1300 rpm for 5 min. After removing the supernatant, the cell pellet was resuspended in 1 mL of Nicoletti Buffer (0.1% of Sodium citrate 0.01% of Tritox-100, 50 µg/mL of Propidium Iodide, 10 µg/mL of RNAse solution) and incubated in the dark at 4 °C for 16 h.

Apoptotic cells were detected by using the CaspGlow Fluorescein Active Caspase 3 Staining kit (Biovision, Milpitas, CA, USA) and Brilliant Violet 421 Annexin V apoptosis staining kit (Biolegend, San Diego, CA, USA) according to the manufacturer's protocol. CR-CSCs were then analyzed using the FACSLyric flow cytometer (BD Biosciences, Franklin Lakes, NY, USA).

3.3.9. Statistical analysis

Data are reported as mean ± SD of results from three independent experiments. Statistical analysis was performed using an analysis of variance test (ANOVA), and multiple comparisons were made with the Bonferroni's test with GraphPad Prism 8.0 software (San Diego, CA, USA). Significance was assumed at $p < 0.05$.

3.4. Results

3.4.1. Polyphenolic Profile of Citrus sinensis and Humulus lupulus Extracts

The study involved the analytical characterization of the main polyphenolic molecules contained in the hydroalcoholic extracts isolated from the juice of *Citrus sinensis* (Figure 26) and from the inflorescences of *Humulus lupulus* (Figure 27). The RP-UHPLC-PDA-IT-TOF analyzes allowed us to identify in the *Citrus sinensis* extract (Table 4) about 19 molecules and in particular flavones such as Apigenin 6,8-di-C-glucoside (Vicenin-2), flavanones such as for example Narirutin and Hesperidin and polymethoxyflavones such as Nobiletin and Tangeretin.

From the LC-MS/MS analysis of the polyphenolic extract isolated from hops (Table 5), about 46 compounds were identified and in particular procyanidins such as Procyanidin b, flavonols such as Quercetin 3-O-glucoside and Kaempferol 3-O-glucoside, flavanones such as Rutin, α-acids, mainly composed of Humulone, Cohumulone and ad-humulone, β-acids and in particular Lupulone, Colupulone and Adlupulone and prenylflavonoids such as Xanthumol.

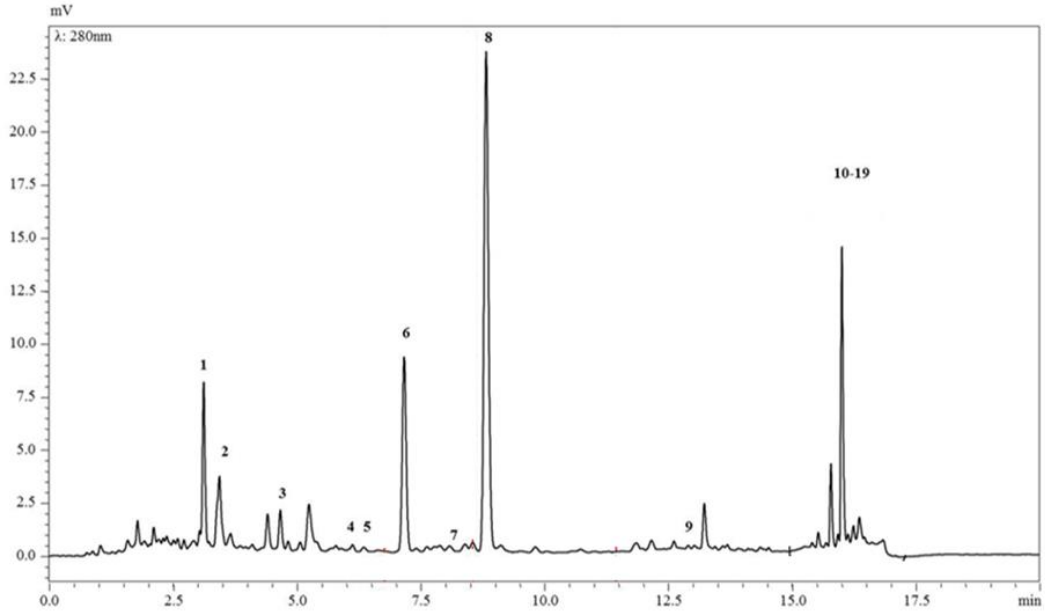


Figure 26. *Chromatographic profile of polyphenols in Citrus sinensis extract.*

Table 4. *Polyphenol profile of extract from Citrus sinensis.*

Peak	Rt (min)	$[M-H]^-$	$[M-H]^+$	MS^2 *m/z*	Error (ppm)	Molecular formula	Compound
1	3.12	593.1518	–	353.0691	1.18	$C_{27}H_{30}O_{15}$	vicenin-2
2	3.44	623.1628	–	383.0791	1.60	$C_{28}H_{32}O_{16}$	lucenin-2 4'-methyl ether
3	4.41	609.1478	–	301.0382	2.79	$C_{28}H_{34}O_{15}$	neohesperidin
4	6.13	595.1692	–	288.9530	4.03	$C_{27}H_{32}O_{15}$	eriocitrin
5	6.35	463.0890	–	301.0353	1.73	$C_{21}H_{20}O_{12}$	isoquercitrin
6	7.17	579.1835	–	271.0636	1.35	$C_{27}H_{32}O_{14}$	narirutin
7	8.08	607.1317	–	300.0320	1.98	$C_{28}H_{32}O_{15}$	neodiosmin
8	8.82	609.1738	–	301.0736	3.28	$C_{28}H_{34}O_{15}$	hesperidin
9	13.23	593.1895	–	285.0763	3.20	$C_{28}H_{34}O_{14}$	didymin
10	15.43	–	373.1236	343.0805	3.48	$C_{20}H_{20}O_7$	isosinensetin
11	15.64	–	403.1371	373.0918	-3.97	$C_{21}H_{22}O_8$	hexamethoxyflavone
12	15.78	–	373.1268	312.0990	3.49	$C_{20}H_{20}O_7$	sinensetin
13	16.07	–	403.1362	373.0923	-0.25	$C_{21}H_{22}O_8$	hexamethoxyflavone *isomer*
14	16.22	–	403.1331	373.0936	0.50	$C_{21}H_{22}O_8$	nobiletin
15	16.25	–	343.1166	282.0876	-2.91	$C_{19}H_{18}O_6$	tetramethyl-*o*-isoscutellarein
16	16.48	–	433.1472	403.1019	-4.85	$C_{22}H_{24}O_9$	heptamethoxyflavone
17	16.67	–	373.1238	343.0809	4.02	$C_{20}H_{20}O_7$	tangeretin
18	16.86	–	389.1215	359.0741	2.06	$C_{20}H_{20}O_8$	hydroxypentamethoxyflavone
19	17.40	–	419.1318	389.0866	-4.53	$C_{21}H_{23}O_9$	3-hydroxynobiletin

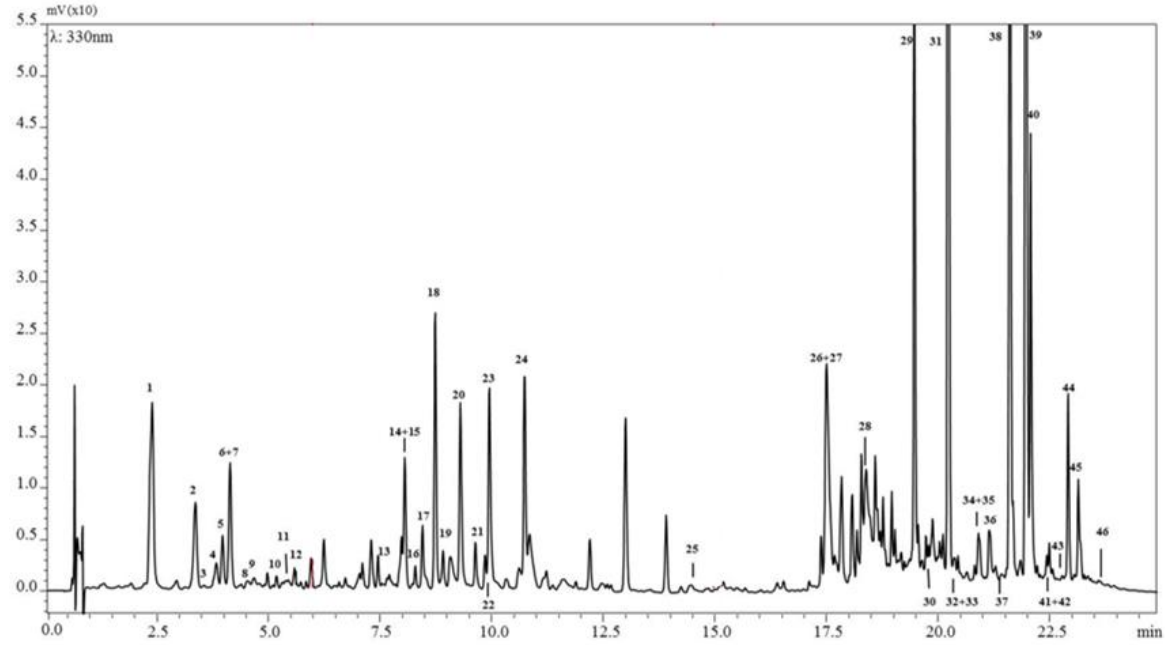


Figure 27. *Chromatographic profile of polyphenolic in Humulus lupulus extract.*

Table 5. *Polyphenol profile of extract from Humulus lupulus.*

Peak	Rt (min)	$[M-H]^-$	MS^2 *m/z*	Error (ppm)	Molecular formula	Tentative identification
1	2.39	353.0860	191.0588/ 179.0345	2.30	$C_{16}H_{18}O_9$	chlorogenic acid
2	3.33	337.0913	191.0573/ 163.0417	-4.75	$C_{16}H_{18}O_8$	3-p-coumaroylquinic acid
3	3.55	577.1354	407.0747/ 289.0692	0.35	$C_{30}H_{26}O_{12}$	procyanidin b
4	3.80	577.1327	407.0737/ 289.0687	0.87	$C_{30}H_{26}O_{12}$	procyanidin b *isomer* (I)
5	3.92	289.0705	245.0810	2.41	$C_{15}H_{14}O_6$	epicatechin
6	4.14	367.1011	193.0580	-6.54	$C_{17}H_{20}O_9$	feruloylquinic acid
7	4.22	865.1949	577.1320/ 407.0740/ 289.0638	-4.16	$C_{45}H_{38}O_{18}$	procyanidin trimer
8	4.50	865.1987	577.1342/ 407.0762/ 289.0635	-2.84	$C_{45}H_{38}O_{18}$	procyanidin trimer *isomer* (I)
9	4.74	577.1384	407.0750/ 289.0707	2.10	$C_{30}H_{26}O_{12}$	procyanidin b *isomer* (II)
10	5.19	337.0951	163.0410	-1.97	$C_{16}H_{18}O_8$	3-p-coumaroylquinic acid *isomer*
11	5.46	865.1912	577.1265/ 407.0751/ 289.0664	-4.16	$C_{45}H_{38}O_{18}$	procyanidin trimer *isomer* (II)
12	5.62	577.1361	407.0794/ 289.0758	3.84	$C_{30}H_{26}O_{12}$	procyanidin b *isomer* (III)
13	7.46	755.2072	489.1013/ 300.0262	3.90	–	quercetin derivate
14	7.99	609.1450	300.0264/ 271.0225	-1.81	$C_{27}H_{30}O_{16}$	quercetin 3-O-rhamnosyl-galactoside
15	8.06	357.1196	195.0672	4.00	–	unknown glucoside

16	8.29	739.2098	385.1357/ 255.0283/ 227.0323	0.95	$C_{33}H_{40}O_{19}$	kaempferol 3-*O*-xylosyl-rutinoside
17	8.48	609.1467	300.0264/ 271.0225	-0.82	$C_{27}H_{30}O_{16}$	rutin
18	8.79	463.0858	301.0322/ 271.0228	-3.89	$C_{21}H_{20}O_{12}$	quercetin 3-*O*-glucoside
19	8.96	593.1507	285.0377/ 255.0290/ 227.0327	-0.84	$C_{27}H_{30}O_{15}$	kaempferol 3-*O*-rutinoside
20	9.33	505.0978	301.0315/ 271.0226	4.12	–	quercetin derivate
21	9.63	593.1571	285.0377/ 255.0290/ 227.0327	-1.52	$C_{27}H_{30}O_{15}$	kaempferol 3-*O*-rutinoside *isomer*
22	9.88	635.1614	285.0353/ 255.0294/ 227.0351	-0.63	$C_{29}H_{32}O_{16}$	Kaempferol 3-*O*-(6"-acetyl-galactoside)-7-*O*-rhamnoside
23	9.94	447.0921	285.0374/ 255.0294/ 227.0354	-2.81	$C_{21}H_{20}O_{11}$	kaempferol 3-*O*-glucoside
24	10.76	489.1035	285.0380/ 255.0296/ 227.0360	-0.61	$C_{23}H_{22}O_{12}$	kaempferol 3-*O*-acetyl-glucoside
25	14.57	347.1865	278.1110	0.29	$C_{20}H_{28}O_5$	cohumulone
26	17.53	317.1747	248.1045/ 180.0470	-3.47	$C_{19}H_{26}O_4$	cohulupone
27	17.66	377.1941	263.1278/ 221.0702	3.59	$C_{21}H_{30}O_6$	humulinone
28	18.48	331.1883	262.1229/ 194.0584	-9.66	$C_{20}H_{27}O_4$	unknown
29	19.48	339.1215	220.3184/ 175.0760/ 151.0789	-6.78	$C_{20}H_{20}O_5$	desmethylxanthohumol
30	19.72	361.1857	265.1435	1.84	$C_{21}H_{30}O_7$	iso-α-ad/n-humulone
31	20.20	353.1368	233.0823/ 189.0913/ 165.0970	-7.36	$C_{21}H_{22}O_5$	xanthohumol
32	20.37	375.1819	306.1051	2.18	$C_{22}H_{32}O_5$	prehumulone
33	20.46	377.1910	263.1278/ 220.0702	-8.22	$C_{21}H_{30}O_6$	humulinone *isomer*
34	20.90	415.2481	302.1504/ 259.0960	-2.17	$C_{25}H_{36}O_6$	lupulone e
35	20.94	333.1700	264.0983/ 221.0472	4.63	$C_{19}H_{26}O_5$	posthumulone
36	21.30	415.2417	371.2200/ 302.1245/ 259.0960	-2.17	$C_{25}H_{36}O_6$	lupulone e *isomer*
37	21.38	415.2465	330.1585/ 287.1309	5.00	–	unknown
38	21.66	347.1847	278.1140/ 235.0610	-4.90	$C_{20}H_{28}O_5$	cohumulone *isomer*
39	21.93	361.2010	343.1893/ 292.1299	-2.77	$C_{21}H_{30}O_5$	n-humulone
40	22.15	361.2010	292.1327/ 235.0679	-9.41	$C_{21}H_{30}O_5$	ad-humulone
41	22.52	375.2158	306.1431/ 263.0888/ 251.0748	-5.06	$C_{22}H_{32}O_5$	prehumulone *isomer*
42	22.56	385.2370	316.1645/ 273.1123/ 248.1056	-3.63	$C_{24}H_{34}O_4$	postlupulone

43	22.81	519.2587	450.1890/ 406.1875	8.86	$C_{35}H_{36}O_4$	unknown
44	22.88	399.2519	287.1260/ 275.1290/ 262.1204	-5.51	$C_{25}H_{36}O_4$	colupulone
45	23.12	413.2680	301.1427/ 289.1449/ 276.1364	3.18	$C_{26}H_{37}O_4$	adlupulone
46	23.46	427.2860	315.1563/ 290.1537	1.40	$C_{27}H_{40}O_4$	prelupulone

3.4.2. Fractionation of Citrus sinensis and Humulus lupulus Extracts

In order to purify the extracts and therefore to obtain fractions with potential onco-nutraceutical interest, a fractionation of the plant extracts was performed by semi-preparative liquid chromatography in reverse phase based on the polyphenolic profile of the extracts of *Citrus sinensis* and *Humulus lupulus* and considering the order of elution of the molecules of interest.

Fractionation of polyphenolic extracted from *Citrus sinensis* led to the collection of four different fractions (Figure 28): fraction I was constituted of peaks 1 to 5, while narirutin and neodiosmin (peaks 6 and 7 respectively) were collected as fraction II. The region III was composed by hesperidin and the fraction IV was constituted by didymin and polymethoxyflavones.

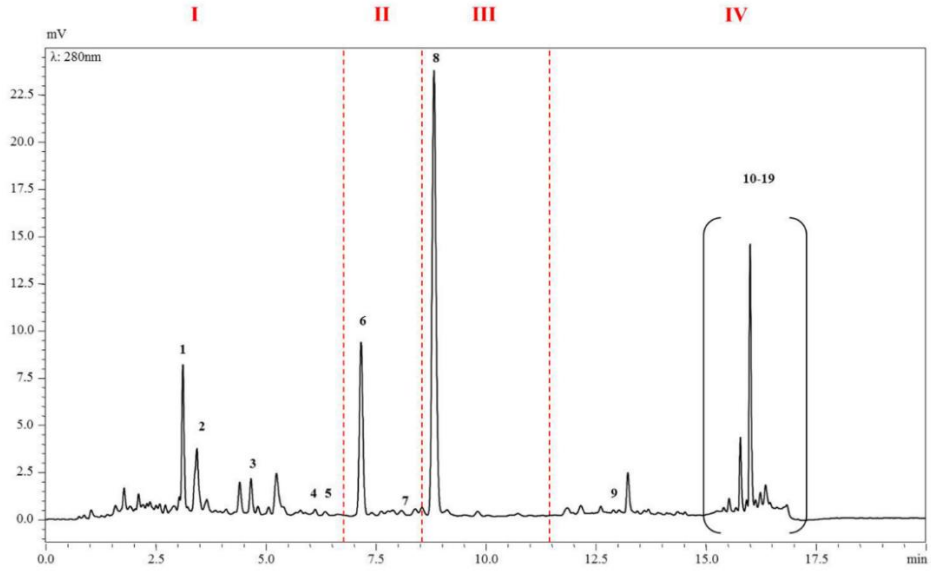


Figure 28. *Fractionation of the polyphenolic profile of Citrus sinensis extract with Semiprep-HPLC.*

The chromatogram of *Humulus lupulus* extract has been divided into three fractions (Figure 29): the first fraction was constituted of procyanidins (peaks 1 to 12), while the fraction II was composed by polyphenolic compounds (peaks 13-25). The region III showed 20 peaks corresponding to the alpha and beta acids. Finally, the fractions were lyophilized for 24 h at -52°C (LyoQuest-55, Telstar Technologies, Spain).

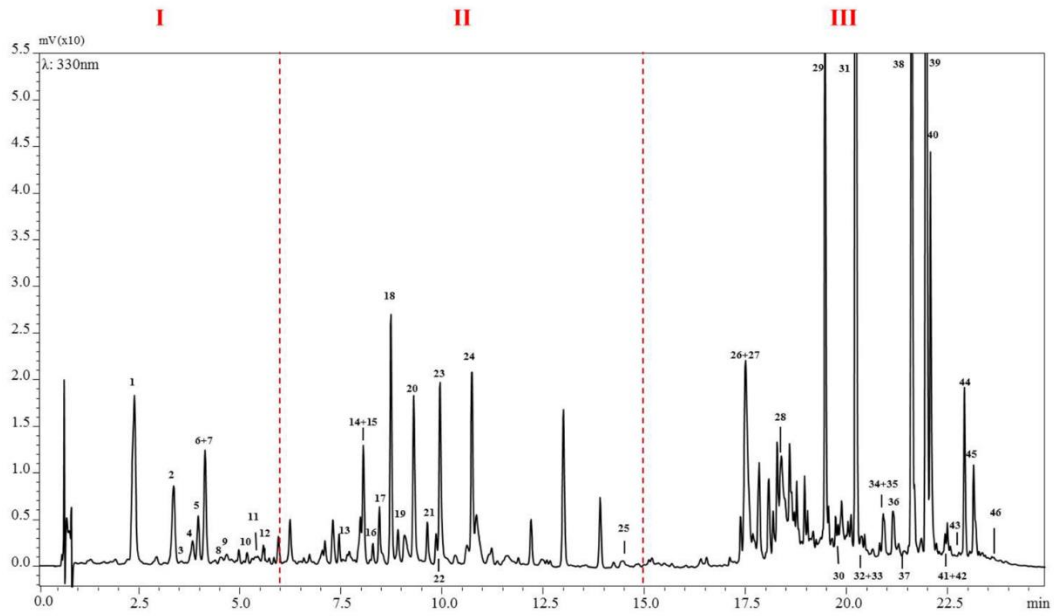


Figure 29. *Fractionation of the polyphenolic profile of Humulus lupulus extract with Semiprep-HPLC.*

From the study of the literature, it emerged that both matrices have numerous biological properties, including antioxidant, anti-inflammatory, and in particular anticancer properties, attributable to the two main groups of compounds, i.e., the fraction of polymethoxyflavones (Figure 30) and prenylflavonoids (Figure 31), respectively fractions IV and III of orange and hops, identifying Nobiletin and Xanthohumol as the majority components.

In detail, compounds **3** and **5** of the polymethoxyflavones contained in the NCF fraction were identified by comparison with the reference standards, respectively, such as

Nobiletin and Tangeretin. Compound **1** characterized as pentamethoxyflavone was identified as Sinensetin, while chromatographic peaks **2** and **4** were identified as Hexa- and Heptamethoxyflavone, respectively.

Compounds **6**, **7** and **8** had in the mass spectra the ions m/z 367, m/z 369 and m/z 353 $[M-H]^-$ as precursors respectively and as main fragments m/z 247, m/z 249 and a m/z 233 corresponding to ions generated following a retro-Diels-Alder $[M-H-C_8H_8O]^-$. Compounds **6**, **7** and **8** contained in the XCF bioactive extract were identified as 5,7-di-O-methyl-8-prenylnaringenin, Ox-xanthohumol and xanthohumol, respectively. Polyphenolic compounds identified in the isolated fractions are reported in Table 6.

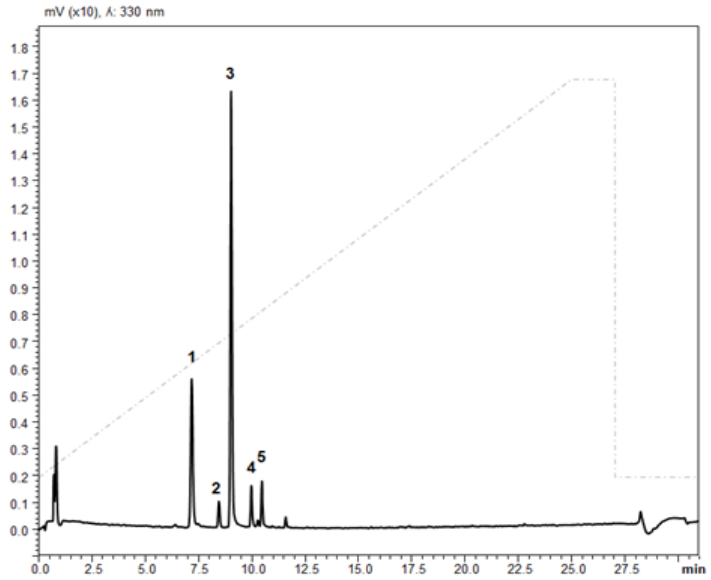


Figure 30. *Chromatographic profile of polymethoxyflavones isolated from Citrus sinensis.*

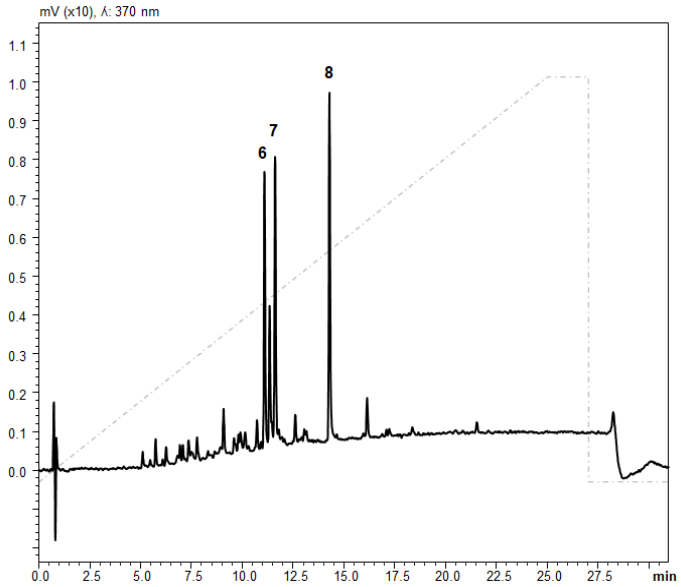


Figure 31. *Chromatographic profile of prenylflavonoids isolated from Humulus lupulus.*

Table 6. *Qualitative profile of the isolated polyphenolic fractions.*

Polyphenolic fraction	Peak	Compound	Molecular formula	[M-H]$^-$	[M-H]$^+$	MS^2 m/z	Error (ppm)
NCF Polymethoxyflavones (*Citrus sinensis*)	1	Sinensetin	$C_{20}H_{20}O_7$	-	373,1337	343,0861	1,88
	2	Hexamethoxyflavone	$C_{21}H_{22}O_8$	-	403,1454	373,0970	1,98
	3	Nobiletin	$C_{21}H_{22}O_8$	-	403,1456	373,0975	2,48
	4	Heptamethoxy flavone	$C_{22}H_{24}O_9$	-	433,1568	403,1089	3,69
	5	Tangeretin	$C_{20}H_{20}O_7$	-	373,1335	343,0866	3,69
XCF Prenylflavonoids (*Humulus lupulus*)	6	5,7-Di-*O*-methyl-8-Prenylnaringenin	$C_{22}H_{24}O_5$	367,1238	-	247,0667	5,3
	7	Ox-Xanthohumol	$C_{21}H_{22}O_6$	369,1392	-	249,0805	4,8
	8	Xanthohumol	$C_{21}H_{22}O_5$	353,1434	-	233,0838	4,0

3.4.3. Cytotoxic Activity of Nobiletin and Xanthohumol on CR-CSphC Sparing Healthy Cells

Polymethoxyflavones (NCF) and prenylflavonoids (XCF) and their combination (Mix) were initially tested on two healthy cell lines, in order to evaluate their possible impact on viability and in particular on human stromal cells, HS-5, and human umbilical vein endothelial cells, HUVEC. These cell lines have been widely described as reliable models for estimating and predicting the side effects of anticancer drugs on healthy cells

(May et al., 2018, Henrique et al., 2020). The two cell lines were then treated with increasing concentrations (5, 10 and 25 µg/mL) of NCF, XCF or their Mix for 24 and 48 hours. At the end of the experiments, only slight effects on cell viability were observed (Figure 32), supporting the absence of significant toxicity of the bioactive fractions investigated and therefore could serve as potential adjuvants of anticancer compounds, minimizing the occurrence of unwanted side effects.

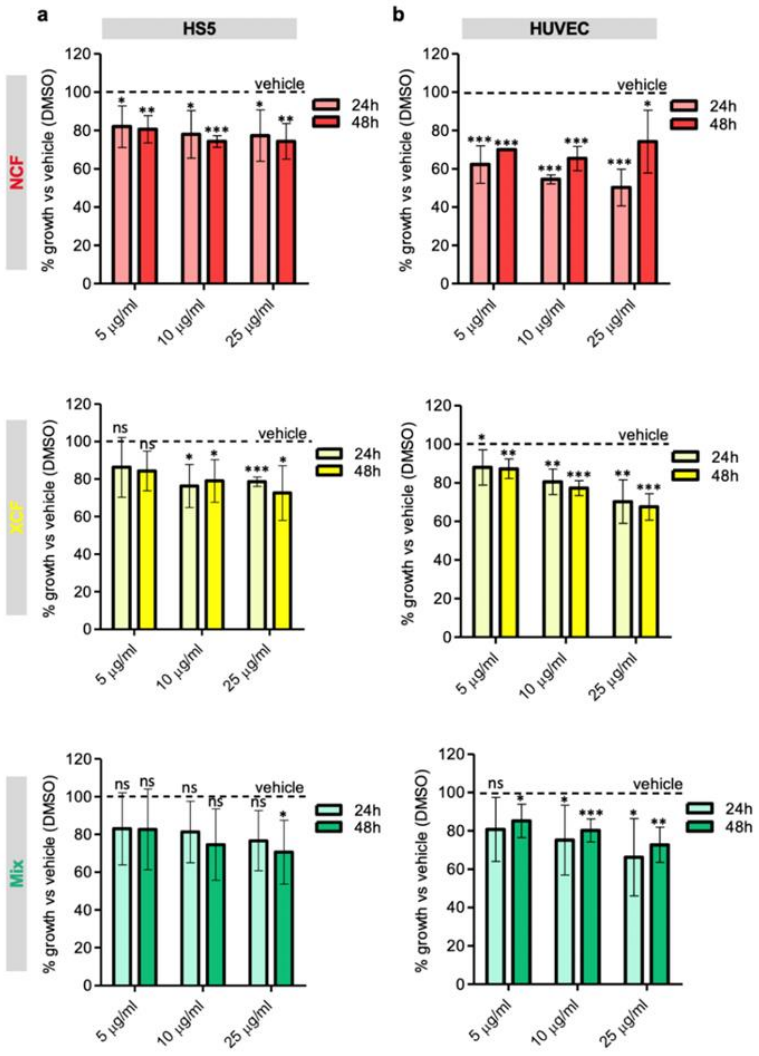


Figure 32. *NCF and XCF do not affect non-transformed cells Percentage of growth of HS-5 (**a**) and HUVEC (**b**) cell lines treated with 5, 10, and 25 µg/mL of NCF, XCF, or the Mix of extracts for 24 and 48 hours. Values are plotted as the percentage of growth versus the vehicle (DMSO, dotted line). Data are represented as means ± SD. Comparisons between two groups*

*(cells treated with the extracts vs cells treated with the vehicle) were made using a two-tailed Student's t-test: *p≤0.05, **p≤0.01, ***p≤0.001.*

In order to assess the potential use of either NCF, XCF or Mix as chemo-sensitizing agents, the potential synergistic effects of these natural compounds with 5-fluorouracil and oxaliplatin (FOX) on CR-CSphCs, isolated from both naïve primary CRC and liver metastasis of patients refractory to chemotherapy, were examined. CR-CSphCs cells are a subpopulation of spheroidal colorectal cancer cells with stem cells characteristics, commonly identified through the expression of specific surface markers such as CD133 and CD44v6, is endowed with peculiar stem cell properties (Todaro et al., 2014). This cell line has a high ability to resist chemotherapy and is responsible for the recurrence of tumors and the formation of metastases (Todaro et al., 2007).

In particular, selected three concentrations of extracts, 12.5 µg/mL, 25 µg/mL and 40 µg/mL, to test their effects on CR-CSphCs proliferation. The administration of NCF, XCF and their Mix significantly reduced the proliferation of CR-CSphCs, including chemotherapy-resistant cells (Figure 33a-b and Figure 34). Combined exposure to NCF and FOX reduced the viability of six CR-CSphCs (#8, #24, #R1, #R2, #R3 and #R4), while treatment with FOX in combination with XCF, significantly reduced viability of four CR-CSphCs (#R7p, #24, #37 and #R2) (Figure 33a and Figure 34a-d). Specifically, the CR-CSphCs subpopulation (#24) was treated with 25 or 40 µg/mL of NCF, XCF or Mix extracts, alone or in combination with 1.25 or 5 µM FOX for 48 hours, while the CR-CSphCs cells (#8) were treated with 12.5 or 25 µg/mL of NCF, XCF or Mix extracts, alone or in combination with 0.5 or 2.5 µM FOX for 48 hours.

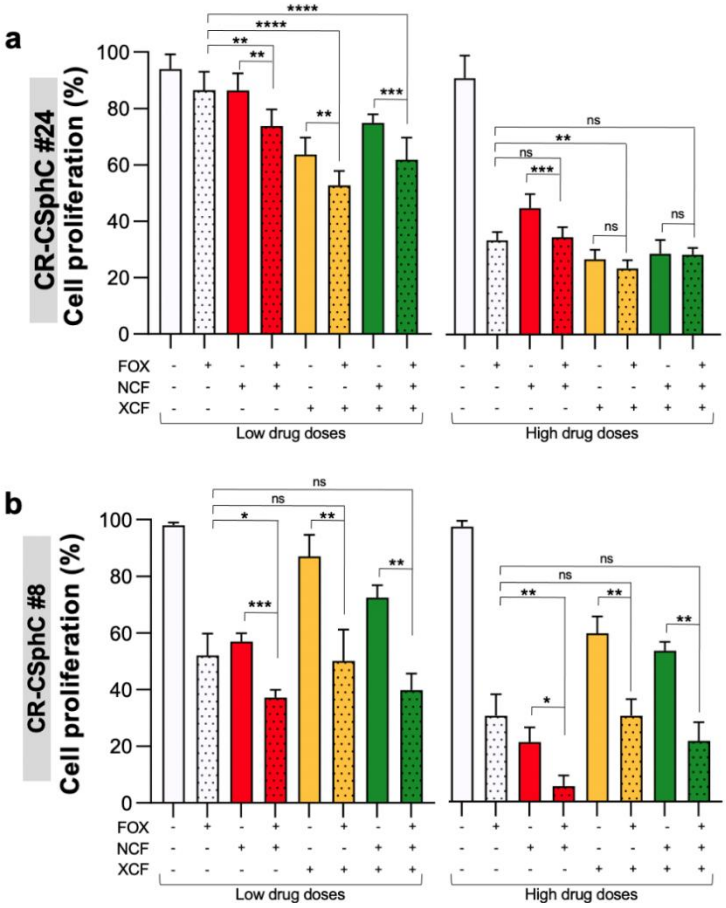


Figure 33. *Combination of NCF plus chemotherapy hamper the proliferation of CR-CSphCs. (a) Cell proliferation percentage of CR-CSphCs (#24) treated with 25 or 40 µg/mL of NCF, XCF or Mix extracts alone or in combination with 1.25 or 5 µM FOX for 48 hours. Data are represented as mean ± SD of three independent experiments. Comparisons between two groups were made using a two-tailed Student's t-test: ns, not significant, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001; (b) Cell proliferation percentage of CR-CSphCs (#8) treated with 12.5 or 25 µg/mL of NCF, XCF or Mix extracts alone or in combination with 0.5 or 2.5 µM FOX for 48 hours. Data are represented as mean ± SD of three independent experiments. Comparisons between two groups were made using a two-tailed Student's t-test: ns, not significant, *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.*

CR-CSphC cells (#R7p) were treated with 25 and 40 µg/mL of NCF, XCF or Mix extracts alone or in combination with 5 µM FOX for 72 hours, while CR-CSphC cells (#R37) were treated with 25 µg/mL of NCF, XCF or Mix extracts alone or in combination with 1.25 µM FOX for 72 hours. CR-CSphCs #R1, #R2 and #R3 cells were treated with 40 µg/mL of NCF, XCF or Mix extracts alone or in combination with 5 µM FOX for 72

hours, while CR-CSphCs #R4 and #R7 treated with 25 µg/mL of NCF, XCF or Mix extracts for 72 hours.

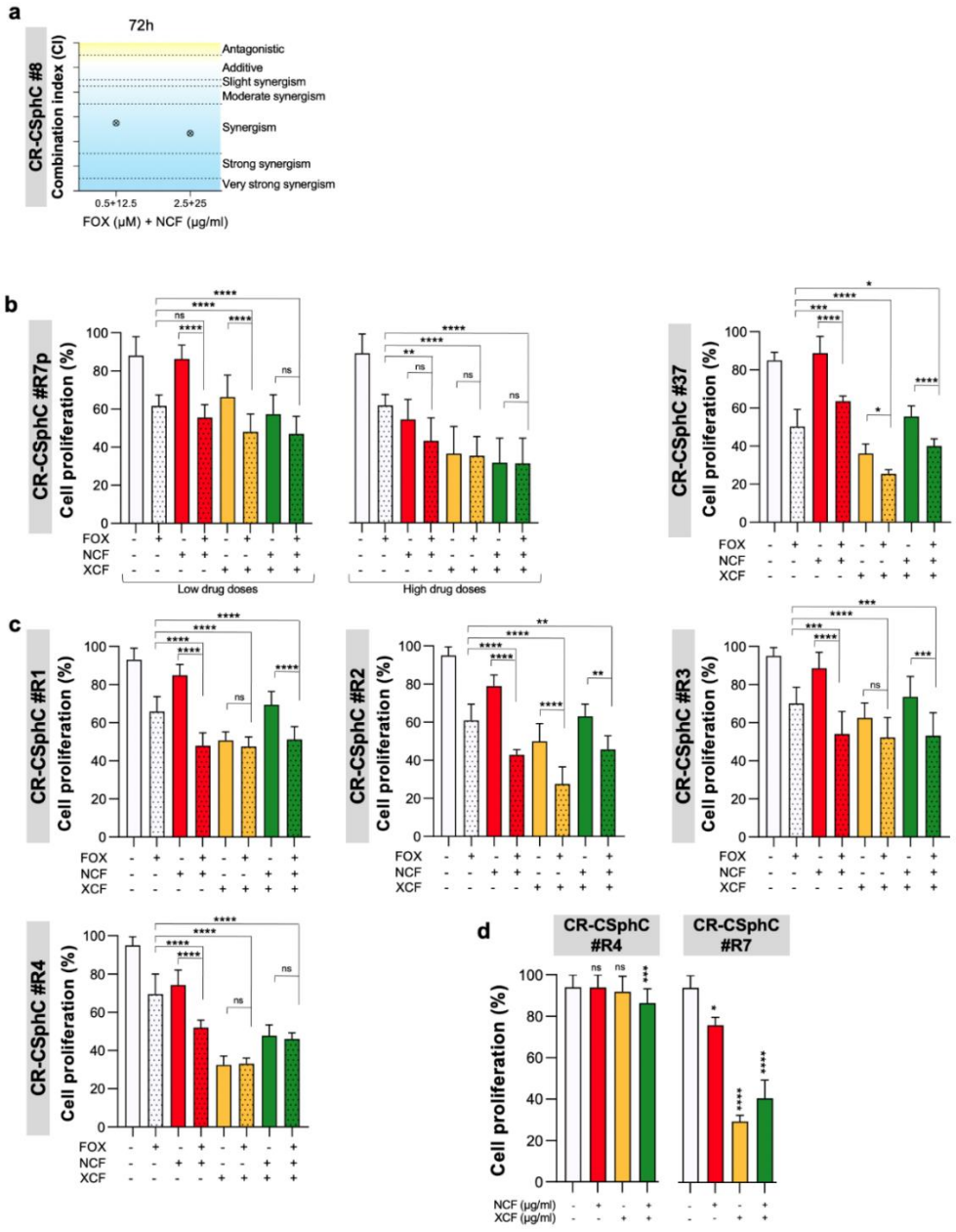


Figure 34. *NCF and XCF sensitize cancer cells to chemotherapy. (**a**) Synergy plot representing the combination index (CI), computed in CompuSyn by using Chou-Talalay method, calculated from cell proliferation data of CR-CSphCs (#8) treated with different FOX and NCF dose pair at 72 hours; (**b**) Cell proliferation percentage of primary CR-CSphCs (#R7p) treated with 25 and 40 µg/mL of NCF, XCF or Mix extracts alone or in combination with 5 µM FOX for 72 hours (left panel) and CR-CSphCs (#R37) treated with 25 µg/mL of NCF, XCF or Mix extracts alone or in combination with 1.25 µM FOX for 72 hours (right panel); (**c**)*

*Cell proliferation percentage of CR-CSC #R1, #R2, #R3, #R4 treated with 40 µg/mL of NCF, XCF or Mix extracts alone or in combination with 5 µM FOX for 72 hours; (**d**) Cell proliferation percentage of CR-CSC #R4 and #R7 treated with 25 µg/mL of NCF, XCF or Mix extracts for 72 hours. Data are represented as mean ± SD of three independent experiments. Percentage of untreated control (vehicle) is shown. Comparisons between two groups were made using a two-tailed Student's t-test: ns, not significant, *p≤0.05, **p≤0.01, ***p≤0.01.*

The analysis of the combination index (CI), calculated with the Chou Talalay method, highlighted the synergistic effects of the NCF fraction and the Mix with FOX in reducing the viability of CR-CSphC (#8) (Figure 34a and 35).

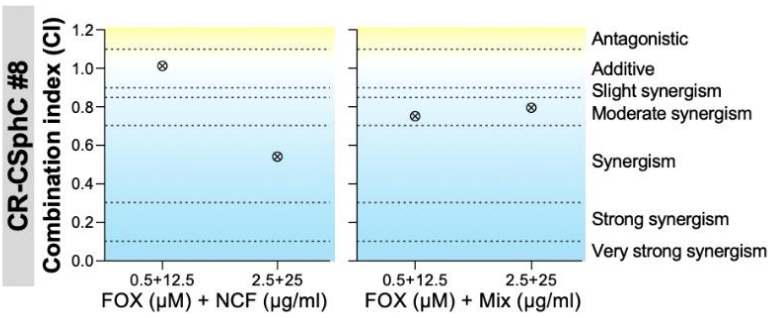


Figure 35. *Synergy plot representing the combination index (CI), calculated in CompuSyn using the Chou-Talalay method, calculated from cell proliferation data of CR-CSphC (#8) treated with different dose pairs of FOX and NCF and Mix at 48 hours.*

These results were also validated on CRC cell lines (HCT116 and RKO) treated with 25 µg/mL of NCF, XCF or Mix extracts alone or in combination with 5 µM FOX at 24 hours, 48 hours and 72 hours. The results showed a significant decrease in cell proliferation following the administration of the XCF, NCF and Mix fractions and the combined treatments FOX-XCF and FOX-NCF (Figure 36a-b).

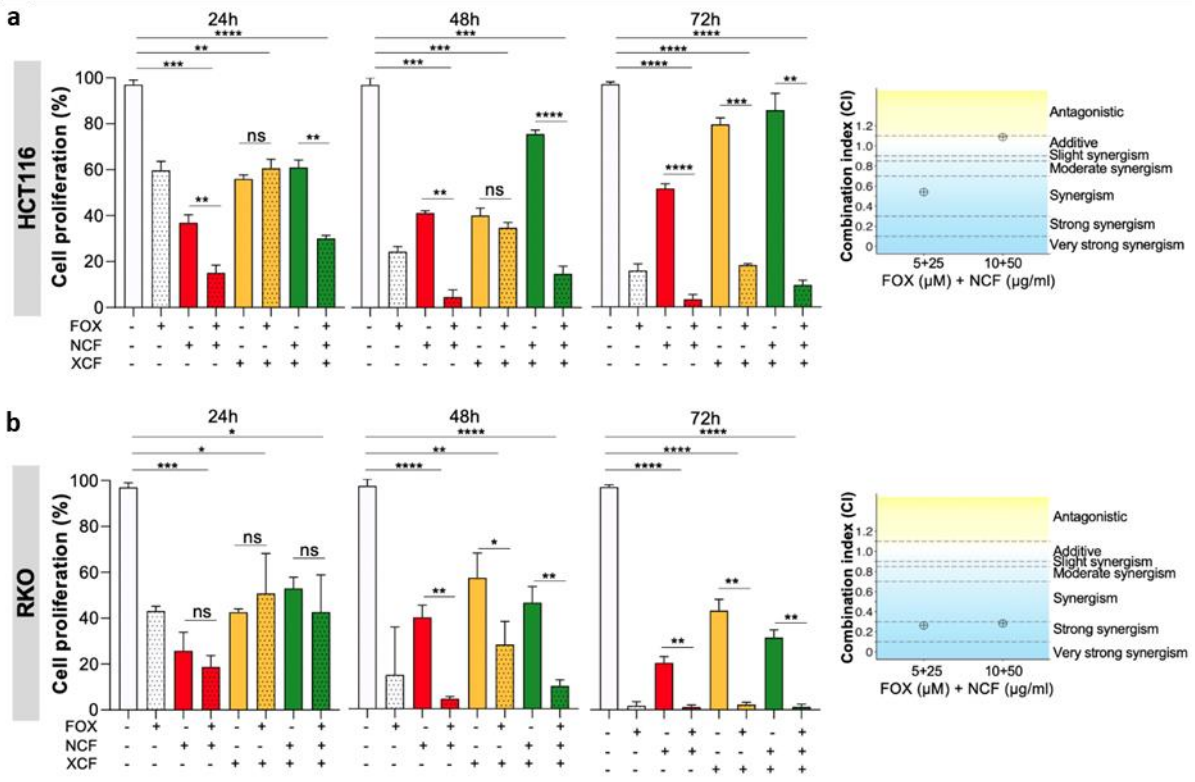


Figure 36. *Cell viability of CRC cell lines (HCT116 (**a**) and RKO (**b**)) treated with 25 μg/mL of NCF, XCF or Mix extracts alone or in combination with 5 μM FOX at the indicated time points (**left panel**). Synergy plot representing the combination index (CI), computed in CompuSyn by using Chou-Talalay method, calculated from cell proliferation data of CRC cell lines treated with different FOX and NCF dose pair at 48 hours (**right panel**). Data are represented as mean ± SD of three independent experiments. Percentage of untreated control (vehicle) is shown. Comparisons between two groups were made using a two-tailed Student's t-test: ns, not significant, *p≤0.05, **p≤0.01, ***p≤0.01.*

3.4.4. Nobiletin and Xanthohumol Induce Apoptosis of CR-CSphCs in Combination with Chemotherapy

The genotoxic stress dictated by the extracts plus chemotherapy caused a reduction in CR-CSphCs S- and G2/M- cell cycle phases and substantially increased the G0/G1 and sub-G0 phase (Figure 37a and Figure 38a).

The cytostatic effect caused by the combined treatment is conceivably induced by the short-term exposure of 48 hours, which concomitantly allows early events of apoptosis. Accordingly, the combination of treatments induced the apoptotic events in CR-CSphCs,

as demonstrated by increased number of Annexin V-positive cells (Figure 37 and Figure 38b). To further confirm that NCF, XCF, and Mix are required to induce programmed cell death in combination with chemotherapy in CR-CSphCs, the expression of genes related to apoptosis and autophagy were analyzed. Gene expression analysis revealed enhanced expression of 8 proapoptotic genes, ATG3, ATG5, ATG12, B2M, CD40, CYLD, FAS, GADD45A, up-regulated in CR-CSphCs treated with 0.5 μM FOX and 12.5 μg/mL NCF, XCF or both extracts (Mix) for 48 hours (Figure 37c).

Polyphenolic extracts have been found to be able to induce apoptosis by increasing the expression of genes associated with apoptosis without determining a significant increase in Caspase-3 activity (Figure 38b), likely due to a caspase-3 independent apoptosis phenomenon. These results suggest that fractionated extracts lessen the common dose of chemotherapeutic drugs, thereby reducing their side effects and rendering more acceptable the therapeutic regimen.

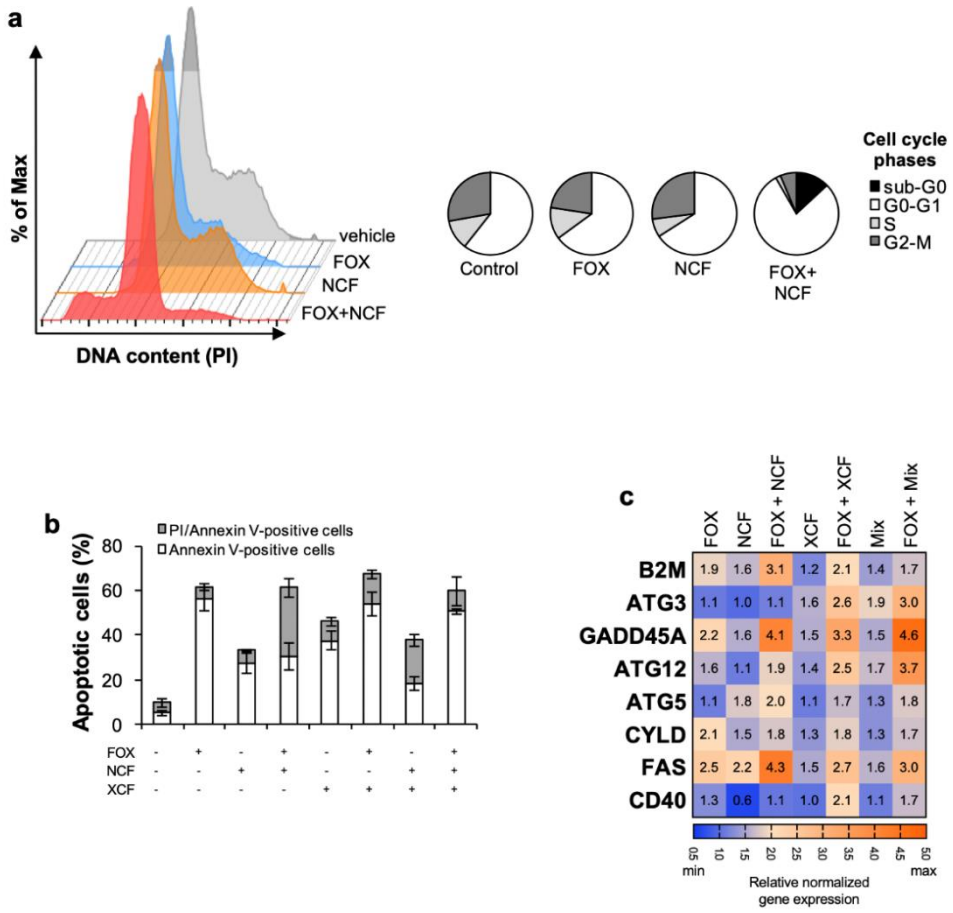


Figure 37. *Flavonoids extracts plus chemotherapy induce apoptosis in CR-CSphCs. (**a**) Phase Representative flow cytometry analysis of cell cycle phases distribution in CR-CSphCs exposed to 0.5 µM FOX and 12.5 µg/mL NCF, alone or in combination, for 48 hours. DNA content was assessed by propidium iodide (PI) staining; (**b**) Percentage of apoptotic CR-CSphCs (#8) treated with 0.5 µM FOX and 12.5 µg/mL NCF, XCF or both extracts, alone or in combination, for 48 hours. The analysis was performed by flow cytometry on CR-CSphCs labeled with propidium iodide (PI) and Annexin-V; (**c**) Gene expression analysis of pro-apoptotic genes in CR-CSphCs (#8) after exposure to 0.5 µM FOX and 12.5 µg/mL NCF, XCF or both extracts (Mix), as compared to control (vehicle) for 48 hours. Data are expressed as $2^{-\Delta\Delta Ct}$ expression values normalized to GAPDH and HPRT genes.*

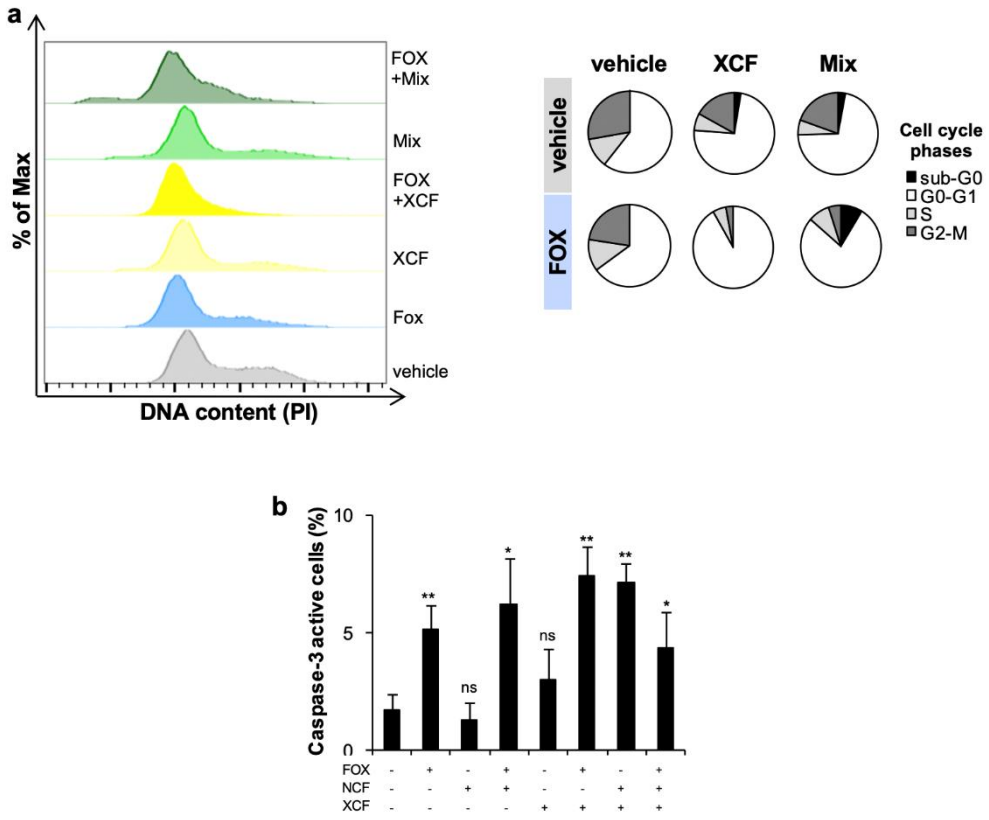


Figure 38. *NCF and XCF plus chemotherapy induce apoptosis in CR-CSphCs. (**a**) Representative flow cytometry analysis of cell cycle phases distribution in CR-CSphCs (#8) exposed to 0.5 µM FOX and 12.5 µg/mL Xanthohumol or Mix, alone or in combination, for 48 hours. DNA content was assessed by propidium iodide (PI) staining; (**b**) Percentage of cells showing caspase-3 activity assessed by flow cytometry analysis.*

3.5. Discussion

The pharmacological use of chemotherapy as a cancer remedy has been limited by its known side effects caused by toxicity to normal cells. Therefore, avoiding unwanted toxicity without compromising targeting of tumor cells represents the main goal to be achieved in cancer treatment. Natural bioactive molecules are known to exert their tumor-preventive effects via antioxidant polyphenols such as flavonoids (Tung et al., 2019, Scagliarini et al., 2020). Flavonoids as natural compounds play a crucial role in preventing damage to the intestinal barrier by preserving its integrity and mucosal

architecture (Wen et al., 2020), suggesting that they may have no side effects on normal intestinal stem cells.

Both bioactive molecules Nobiletin and Xanthohumol, individually or 50% mixed, synergize with FOX-based chemotherapy in order to reduce the vitality of CR-CSphC cells, S phase and G2/M phase of the cell cycle. NCF and XCF in combination with FOX result in an upregulation of pro-apoptotic genes, as well as a downregulation of anti-apoptotic genes. Furthermore, given that many of the pro-apoptotic genes induced by the combined treatment of flavonoids and FOX, such as ATG3, ATG5 and ATG12, play critical roles in the autophagic process (Zhuang et al., 2016), we hypothesize that CR-CSphCs exposed to the combined treatment are experiencing a severe intracellular stress, leading to apoptosis which can follow an autophagic process, without causing a significant increase in Caspase-3 activity. Interestingly, both NCF and XCF, although showing a cytotoxic effect towards tumor cells, are well tolerated by normal cells. This phenomenon is still not fully understood and could be explained by the presence of an altered metabolism or by the activation of molecular pathways exclusively in tumor cells.

In summary, we investigated how NCF and XCF interfere with cell proliferation and apoptosis. Interestingly, these natural plant-derived compounds, singularly or in combination, are useful as additive molecules to chemotherapy, possibly limiting antitumor cytotoxicity towards normal cells. The novelty of our research is based on the synergistic/additive effect of Nobiletin/Xanthohumol treatment in combination with chemotherapy, affecting the viability of purified CR-CSCs propagated from CRC liver metastases of patients treated with chemotherapy.

3.6. Conclusions

In conclusion we investigated the potential effects of natural flavonoids, both polymethoxyflavones and prenylflavonoids, as potential adjuvants in CRC therapy.

Results obtained showed how specific fractions from natural extracts are able in improving the efficacy of chemotherapy, reducing, at the same time, cancer cell survival and chemotherapeutics cytotoxicity. Hence, they suggest that the fractionated extracts of the two matrices could be used as adjuvants of standard anticancer compounds, when used in the form of functional foods and/or dietary supplements. Flavonoids could serve as a promising strategy for anticancer treatment, which preserves patients' quality of life. Specifically, these fractions are characterized by the presence of active compounds that have been previously characterized for their anticancer potential, namely Nobiletin form *Citrus sinensis* and Xanthohumol from *Humulus lupulus*.

CHAPTER IV:

Mediterranean Area: ***Malpighia emarginata, Arbutus unedo, Lycium barbarum l., Annona cherimola, Diospyros kaki***

4. Evaluation of the Potential Biological Properties of Phytocomplexes Extracted from Mediterranean Area Fruits

4.1. Introduction

Cancer is a leading cause of death worldwide and a major barrier to increasing life expectancy in every country in the world (Huang et al., 2009). There were an estimated 19.3 million new cancer cases and 10.0 million cancer deaths in 2020 worldwide. There is about a 20% lifetime risk of getting cancer (before age 75) and a 10% risk of dying from cancer. Breast cancer is the most commonly diagnosed cancer in the world, followed by lung cancer. The most common cause of cancer death remains by far lung cancer, followed by liver and stomach cancer. Prostate cancer was the most frequently diagnosed cancer in males, followed by lung cancer, non-melanoma skin cancer (NMSC), lip and oral cavity, and liver cancer (Ferlay et al., 2021). In females, breast cancer was the most frequently diagnosed cancer in all regions of the world accounting for 11.7% of all total cases and with 2.3 million new cases. It is the fifth leading cause of cancer death worldwide with mortality rate of 685,000 deaths and 6.9% of total cancer deaths (Sung et al., 2021). In recent years, several treatment strategies have been explored in addition to surgery, radiotherapy, and chemotherapy. In fact, chemotherapy is the standard treatment for many types of cancer. However, many chemotherapeutic agents produce adverse side effects and potentially irreversible chronic toxicity (Miller et al., 2016). Therefore, new therapeutic strategies and chemotherapy candidates need to be urgently explored.

Nowadays, great attention has been paid to natural compounds in fruits and vegetables with potential nutraceutical properties. In this regard, it has been widely demonstrated that dietary polyphenols are able not only to reduce oxidative and inflammatory stress, but also to decrease the proliferation of cancer cells (Pepe et al., 2020). However, the biological activity of various food plants has not yet been studied. Numerous studies have focused their attention on the antioxidant, antitumor and anti-inflammatory effects of natural polyphenols, as well as on their ability to reduce oxidative stress, important factor in the cancer development (Li et al., 2014, Shi et al., 2015, Rigalli et al., 2016).

Cancer cells are subjected to oxidative stress, defined as an imbalance between pro-oxidants and antioxidants with concomitant disruption of redox circuits and macromolecular damage (Patel, 2016). Oxidative stress generates numerous molecular species, including reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive lipid species, and free radicals (Liu et al., 2020b). In particular, cancer cells have been shown to have a higher ROS level than normal cells (Tafani et al., 2016). ROS are a heterogeneous group of highly reactive ions and molecules derived from molecular oxygen (O_2) (Pepe et al., 2017). ROS, depending on the concentration and duration of exposure, can induce DNA damage leading to the activation of oncogenes or the inactivation of tumor suppressor genes (Tafani et al., 2016). This can be considered one of the mechanisms by which ROS can promote carcinogenesis. In addition to causing significant genetic changes, ROS can induce tumorigenesis by mediating various epigenetic alterations. Methylation and inactivation of tumor suppressor genes is the most common epigenetic alterations in oxidative stress-induced tumorigenesis (Galadari et al., 2017). Furthermore, ROS can modulate the activities of different proteins or the signaling pathways involved in the proliferation of cancer cells. It has been shown that ROS are critical regulators of angiogenesis, through the activation of pro-angiogenic factors, such

as vascular endothelial growth factor (VEGF), in the tumor microenvironment. A growing body of evidence suggests that ROS are crucial in the processes that regulate tumor cell invasion and metastasis (Fukai and Ushio-Fukai, 2020).

Besides the mitochondrial electron transport chain, which is the major endogenous source of ROS in most mammalian tissues, another important endogenous source of controlled ROS are enzymes such as NADPH oxidase (Nox) (Sies and Jones, 2020). The NADPH oxidase family consists of 7 members (Nox1, Nox2, Nox3, Nox4, Nox5 and the dual oxidases Duox1 and Duox2) with a wide tissue distribution and mechanism of activation (Schröder, 2020). Their subcellular distribution varies in different cell types, from the plasma membrane to the intracellular compartments and the nuclear membrane (Moloney et al., 2017, Cattaneo et al., 2016). NADPH oxidases can be classified according to their mode of activation and ROS production. The first group includes Nox1, Nox2 and Nox3 which can be activated through the assembly of cytosolic subunits and produce $O_2^{\bullet-}$. Nox5, Duox1 and Duox2 belong to the second group, can be activated by Ca^{2+} independently of cytosolic subunits and produce $O_2^{\bullet-}$ or H_2O_2 (Schröder, 2020). The only member of the third group is Nox4, which produces H_2O_2 directly independent of cytosolic subunits and is constitutively active (Abdel-Wahab et al., 2019). In cancer cells, the expression of Nox proteins at the tissue level is highly diverse. Nox1 is most highly expressed in colorectal adenocarcinoma (Rokutan et al., 2008), but is also expressed at lower levels in vascular smooth muscle cells (VSMCs), endothelial cells, uterus, placenta (Cui et al., 2006), prostate and osteoclasts (Lee et al., 2005). Nox2 is most highly expressed in hematopoietic and lymphoid cancers (Bedard and Krause, 2007), while Nox3 in the inner ear led (Paffenholz et al., 2004). Nox4 is highly expressed in the kidney, ovary, brain, and bladder cancer (Geiszt et al., 2000), and Nox5 in prostate cancer and malignant melanoma (Brar et al., 2003). Duox1 is highly expressed in the thyroid and

head and neck cancer, while Duox2 is expressed in breast, colorectal, gastric, lung, prostate, and pancreatic cancer (Konaté et al., 2020). Numerous studies have highlighted the relationship between an overexpression of Nox family genes and formation of an oxidized microenvironment that has a profound impact on tumourigenesis, tumor progression and cell proliferation (Lu et al., 2012, Faria and Fortunato, 2020, Grauers Wiktorin et al., 2020). Previous studies have demonstrated the overexpression of NADPH oxidases in breast cancer, suggesting that H_2O_2 production by NADPH oxidase Nox4, protects cancer cells from cell death, promotes carcinogenesis, increases invasion, growth and EMT (Boudreau et al., 2012, Graham et al., 2010, Choi et al., 2016, Parascandolo and Laukkanen, 2019). This suggests the use of MCF-7 cells for our study. Although further studies are needed to unequivocally link Nox-mediated ROS to cancer, it can be argued that inhibition of tumor growth through selective targeting of NOX is possible, due to the action of Nox-produced ROS in regulating cell division, survival and angiogenesis in cancer.

Phenolics, including polyphenols, flavonoids, anthocyanins, and carotenoids are groups of compounds that exist naturally in plants that have significant antioxidant activity (Saitta et al., 2002). Additionally, they have been associated with the health benefits of consuming large amounts of fruits and vegetables. Their regular consumption has been related to a reduced risk of chronic diseases related to oxidative stress, including neurodegenerative, cardiovascular and coronary diseases and cancer. Phenolics and carotenoids can prevent oxidative damage due to their ability to inhibit ROS but under certain conditions, including high pH, high phenolic concentrations, and the presence of redox-active transition metals such as Fe^{3+} and Cu^{2+} (present in high amounts in cancer cells), phenolic compounds show pro-oxidant activity (Procházková et al., 2011, Park and M Pezzuto, 2012). The direct pro-oxidant activities of phenolic compounds are based on

the generation of a phenoxy radical or a redox complex with a transition metal ion. Phenoxy radicals can react with oxygen to generate $O_2^{\cdot}$, H_2O_2 and a complex combination of semiquinones and quinones (Hodnick et al., 1988). It is assumed that the pro-oxidant activity of several phenolic compounds can induce lipid peroxidation, DNA damage and apoptosis in cancer cells (Zheng et al., 2008). The number of hydroxyl groups in the aromatic rings affects the prooxidant activities of the phenols; the greater the number of hydroxyl substitutions (particularly in the ortho position), the stronger the pro-oxidant activities. Quercetin and myricetin having two and three hydroxyl groups in the ortho position have been shown to exhibit the most potent prooxidant activity among other phenols including meta and para dihydroxy and monohydroxy phenolics. However, it is evident that prooxidant compounds increase cellular levels of ROS to cytotoxic levels in tumor cells but not in normal cells. This effect can be explained by the higher concentration of copper ions and the higher metabolic activity found in cancer cells compared to normal cells (Eghbaliferiz and Iranshahi, 2016).

A high state of oxidative stress makes cancer cells vulnerable and can undergo toxic chain reactions, which induce cells to stop proliferating and die. ROS production has also been shown to initiate ER stress (Lin et al., 2008, Santos et al., 2009). The ER is an essential cellular organelle network consisting of a series of tubules through which protein folding, lipid synthesis, transport of cargo protein and calcium store were fine controlled (Choi et al., 2019). When ER functions are impaired due to different physio-pathological phenomena, such as nutrient deprivation, hypoxia, calcium depletion, an accumulation of misfolded protein aggregates occurs and a condition normally referred to as ER stress occurs (Schwarz and Blower, 2016).

The redox state is in fact closely linked to protein-folding homeostasis within endoplasmic reticulum. Disulfide bond formation in the ER lumen is strongly dependent

on redox balance, where this imbalance can disrupt protein folding and cause ER stress. Consequently, ROS-mediated ER stress triggers a cellular stress response, also defined UPR. The activation of the UPR involves three signaling pathways mediated by three transductors PERK, IRE1 and ATF6, which all play vital functions in overall recovery proteostasis. However, when ER stress entity is too strong, UPR directs the cell toward cell death (Seervi et al., 2018, Read and Schröder, 2021).

In this regard, the aim of this study is to evaluate the potential biological properties of the phytocomplex extracted from five fruits of the Mediterranean area such as *Malpighia emarginata*, *Arbutus unedo*, *Lycium barbarum* L., *Annona cherimola*, *Diospyros kaki*.

4.1.1. Malpighia emarginata

Malpighia emarginata (Figure 39), also known as acerola, belongs to the *Malpighiaceae* family. The fruit is known to be one of the richest natural sources of ascorbic acid in the world. The fruit of evergreen acerola shrub is similar to a trilobite cherry and although the sweetness of the fruit varies according to variety, most are quite tart and acidic (Delva and Schneider, 2013).

Figure 39. *Malpighia emarginata fruits.*

Ascorbic acid is one of the most important water-soluble vitamins, essential for the biosynthesis of collagen, carnitine and neurotransmitters. Acerola is a natural source of vitamin C, the content of which ranges from 1000 to 4500 mg/100 g, which is about 50-100 times that of orange or lemon (Almeida et al., 2014). Furthermore, acerola is a source of numerous macro and micronutrients. Glucose, fructose and a small amount of sucrose are the main sugars found in the ripe acerola fruit. Among the organic acids, malic acid is present in greater quantities while citric acid and tartaric acid are present in smaller quantities (Righetto et al., 2005). In addition to containing a high amount of ascorbic acid, the fruit also contains several phytonutrients such as carotenoids, phenols, flavonoids and anthocyanins (Marques et al., 2016) and possesses numerous bioactives properties. The fruit also contains provitamin A, vitamins B1 and B2, niacin, albumin, iron, phosphorus, and calcium (Delva and Schneider, 2013). The physicochemical properties of the acerola fruit, its nutritional value and phytonutrient content depend on various factors, including the places of cultivation, environmental conditions, cultural practices, stage of ripening, processing and conservation. A number of biological activities have been demonstrated using different extracts of acerola and its phytoconstituents. Being acerola a rich source of powerful antioxidants, such as ascorbic acid and other phytonutrients such as phenols, carotenoids, it has good antioxidant activity useful in fighting against various diseases associated with oxidative stress. Furthermore, acerola also demonstrated several other biological activities such as hepatoprotective, anticarcinogen, antihyperglycemic, antigenic and antimicrobial activity (Prakash and Baskaran, 2018).

4.1.2. Arbutus unedo

Arbutus unedo (Figure 40), commonly called strawberry tree, is a very popular species and studied for its gastronomic and medicinal uses. The *Arbutus* genus belongs to the *Ericaceae* family and includes 12 accepted species with different geographical distribution and interest. *Arbutus* is a small evergreen and flowering tree or shrub whose fruits have traditionally been used in fermented and distilled drinks, jellies, jams, marmalades, sauces and vinegars and are also eaten fresh. This plant native to the Mediterranean can be found in southern, western and central Europe, the Canary Islands, Ireland, north-eastern Africa and western Asia, can withstand temperatures down to -12°C and is tolerant of drought (Miguel et al., 2014, Tenuta et al., 2019).

Figure 40. *Arbutus unedo fruits.*

The fruits are widely used for culinary purposes and have a high content of carbohydrates with a significant fraction of fibres, constituting an interesting source of vegetable proteins and lipids. Indeed, the fruit has high amounts of fructose and glucose and low levels of sucrose and maltose, and several minerals in its composition include calcium, potassium, magnesium, sodium, phosphorus and iron (Ruiz-Rodríguez et al., 2011). The composition and concentrations of some compounds such as vitamins, organic

acids and sugars could depend on the ripening stage of the fruits, the season and the geography (Ait Lhaj et al., 2021, Morgado et al., 2018). However, also leaves, roots, wood and honeys have been appreciated not only for their use in the preparation of food and beverages, but also for their content in bioactive compounds (polyphenols, lipids, proteins, secondary metabolites) which are capable of exert relevant biological activities that may help reduce the incidence of various disorders (Morgado et al., 2018). Different chemical compounds with bioactive properties have been identified in different parts of the plant with applications in pharmaceutical, cosmetic and food industries (Migas and Krauze-Baranowska, 2015). Phenolic acids, galloyl derivatives, flavonols, flavan-3-ols and anthocyanins have been identified as major components of fruit (Guimarães et al., 2013). In addition, unsaturated fatty acids (α -linolenic, linoleic and oleic acid) and saturated fatty acids (palmitic acid), vitamin E and vitamin C, as well as different acids such as fumaric, lactic, malic, suberic, citric, quinic acids have been identified and oxalic (Morales et al., 2013). The traditional use of the leaves as a diuretic, urinary antiseptic, depurative and as an antihypertensive is reported in the literature. In fact, several bioactive properties have been described, such as the antioxidant activity of leaf and fruit extracts, as well as antimicrobial activity against some bacteria and moulds, and also anti-inflammatory, anticancer and vasodilatory activities (Martins et al., 2022, Morales, 2022).

4.1.3. Lycium barbarum L.

Lycium barbarum L. (*L. barbarum*) belongs to Solanaceae family, known as Goji berry or wolfberry, traditionally grow in China, Tibet and other parts of Asia, but production has been increasing in recent decades (Kafkaletou et al., 2018), in particularly in Europe (Italy, Romania, Bulgaria, Portugal, Greece, Serbia), North America and Australia (Vidović et al., 2022).

L. barbarum (Figure 41) has been widely used as a traditional medicinal plant or dietary supplement in China for many years. It has also been found in the Mediterranean area with defoliated shrubs and the ripe fruit is between 1 and 2 cm long, has an ellipsoidal shape and a bright red-orange color (Lu et al., 2019).

Figure 41. *Lycium barbarum berries.*

The fruit is the part of the plant mostly used for medicinal purposes. The fruits of the *Lycium* plant contain a variety of bioactive secondary metabolites, as a result, they are used to develop drugs, dietary supplements and functional foods, with many different pharmacological and therapeutic actions such as antioxidant, immunomodulatory, anti-inflammatory, anti-atherogenic, antifibrotic, antimutagenic, anticancer, anti-radiation, anti-fatigue, anti-aging, anti-stress, anti-yeast, cardioprotective and healing activity (Leontopoulos et al., 2017, Kwok et al., 2019). Major constituents of *L. barbarum* fruit include polysaccharides and proteoglycans, carotenoids, vitamins, fatty acids, free amino acids, flavonoids, phenolic acids, and anthocyanins (Potterat, 2010, Georgiev et al., 2019). Large amount of dicaffeoylspermine/spermidine was found in the root barks of wolfberry. It has strong antioxidant, anti-aging, neuroprotective, anti-Alzheimer's activity

and cytotoxicity are beneficial for human health care (Xiao et al., 2019). Wolfberry root is used to treat fever, night sweats, anxiety, gynecological diseases, night sweats, false kidney back pain, palpitations, insomnia, tears and spermatorrhea (Yao et al., 2018). There are numerous potential biological effects of consuming *Lycium* species (Qian et al., 2017), such as antioxidant, anti-inflammatory, anticancer, antiobesity, antidiabetic, and cardiovascular effects (Chen et al., 2021).

Numerous studies suggest that consumption of *Lycium* barbarum fruits could serve as a potential source of natural antioxidant compounds and that *Lycium barbarum* phenolic extracts could be exploited for nutritional pharmaceutical purposes (Teixeira et al., 2023, Byambasuren et al., 2019).

4.1.4. Annona cherimola

Annona cherimola it is one of the traditionally important semi-deciduous plants belonging to the species *Annona* and the family *Annonaceae* in the order Magnoliales. It is commonly known as cherimoya. The plant is richly distributed with vitamins B1, B2 and B3, as well as iron, calcium and phosphorus. The pulp of the fruit is white, fleshy and juicy. Acetogenins obtained from the seeds have numerous pharmacological properties and are widely used as a bio-insecticide (Vanhove and Van Damme, 2013).

Annona cherimola (Figure 42) is a semi-evergreen shrub or small tree. It is widely distributed in tropical or subtropical regions of America, Africa and Asia, also in southern Europe. They can be found and are commercially grown in several regions with mild temperatures around the world, such as Portugal, Italy, Taiwan and Spain (Anaya-Esparza et al., 2017).

Figure 42. *Annona cherimola fruits.*

Annona cherimola fruit is a typical sweet fruit with a high content of macro and micronutrients such as carbohydrates, vitamin C and significant amounts of vitamin B6, thiamine, riboflavin and folate with a modest amount of vitamin A. Predominantly fruit is the main source of thiamine, riboflavin, iron, niacin, phosphorus and calcium. The Annona cherimola fruit also contains numerous essential minerals and above all high quantities of copper, potassium, manganese and magnesium (Albuquerque et al., 2016).

Phytochemical analyzes showed large amounts of phenolic compounds, especially proanthocyanidins, flavonoids and alkaloids (Mannino et al., 2020). Numerous biological properties manifested by the Annona cherimola extracts such as anti-inflammatory, antidiabetic, antimicrobial, antidepressant, antiviral, anticancer, cytoprotective and antioxidant activity (Jamkhande et al., 2017, Durán et al., 2021).

For these reasons, not only the fruit but also the seeds and leaves of the *Annona cherimola* are studied to identify the main bioactive compounds and their potential use as a nutraceutical to safeguard human health.

4.1.5. Diospyros kaki

Diospyros kaki L. (persimmon) is an arboreal-sized plant belonging to the *Ebenaceae* family. Several species have been identified and the *Diospyros kaki* L. species is the most important (Yaqub et al., 2016). Persimmon (Figure 43) is a fruit appreciated in many

countries for its sensory characteristics, excellent nutritional quality, and for its potential for application in various industrial segments (Milczarek et al., 2018). The first cultivations were documented in China to then be exported and cultivated in many regions of the world, including North and South America, Spain, Italy, Australia. Persimmon is a source of many macro and micronutrients, such as carbohydrates, organic acids, phenolic compounds, carotenoids and tannins as the main nutrients present in the fruit, which gives it antioxidant activity, cytotoxic and antidiabetic (Matheus et al., 2022).

Figure 43. *Diospyros kaki.*

The seeds are rich in fatty acids, of which the most important are palmitic, oleic and linoleic acids (Hricová et al., 2011). The peel is a significant source of dietary fiber, vitamin C, phenolic compounds (especially caffeic, p-coumaric, ferulic and gallic acids), carotenoids (β-cryptoxanthin, α-carotene, zeaxanthin, lutein, lycopene and β-carotene) and proanthocyanidins, which promote good antioxidant capacity (Qi et al., 2019).

The pulp is also a source of vitamins such as ascorbic acid and vitamin A, minerals such as calcium and iron, as well as phenolic compounds (ferulic, p-coumaric and gallic acids) and carotenoids (p-cryptoxanthin, lycopene, carotene, zeaxanthin and lutein) (Yaqub et al., 2016). For these characteristics, this fruit offers a remarkable antioxidant activity.

In addition to antioxidant activity, persimmons have numerous health benefits, including the ability to improve eye health, anti-aging activity, anticancer activity, improve digestion, boost the immune system, lower cholesterol, increase metabolism, strengthen bones, enhance cognitive function, lower blood pressure and skin care. They also help the body heal faster, aid in weight loss, reduce inflammation, and increase blood circulation throughout the body. Persimmon proanthocyanidins play an important role in obesity and diabetes (Pachisia, 2020).

4.2. Materials and Methods

4.2.1. Simple Preparation

L. barbarum fruits were freeze-dried by setting the condenser temperature to -52°C and the vacuum value to 0.100 mBar. The powder (1.0 g) was extracted for 20 minutes with 25 mL of EtOH three times (last time overnight) at 40 °C under magnetic stirring at 470 rpm; The extract was centrifuged at 6000 × rpm for 15 minutes at 4°C and the supernatant filtered under vacuum; subsequently the organic solvent was removed by vacuum evaporation.

The next phase of elimination of sugars was carried out using solid-phase extraction (SPE). SPE silica cartridge is prepared with the calibration solution (6 mL MeOH), then with the equilibration solution (6 mL H_2O). The extract was solubilized in 6 mL of H_2O/ethanol (80:20 v/v) and loaded onto the previously conditioned cartridge, then it is washed with water (6 mL H_2O) to remove the sugar and the polyphenolic components are eluted with 12 mL of MeOH + 2% HCOOH. This is all done in a vacuum system. The elution fractions were collected and solvent removed by evaporation under vacuum.

4.2.2. Cell Culture and In Vitro Cytotoxicity Assay for Initial Screening

The human cervical cancer cell line (HeLa) was grown in Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/mL glucose) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Human breast endothelial line (MCF-10A) was grown in 1:1 mixture of DMEM and Ham's F12 medium. The medium was supplemented with 5% horse serum, 2 mM L-glutamine, human recombinant epidermal growth factor EGF (20 ng/mL), insulin (10 µg/mL), cholera toxin (100 ng/mL), hydrocortisone (5 µg/mL), 100 U/mL penicillin, and 10 µg/mL streptomycin. Cells were cultured under a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C.

The cytotoxic effects of the different extracts were evaluated on the cervical cancer cell line HeLa and on the healthy cell line MCF10A by means of the CellTiter 96 AQueous One Solution Cell Proliferation Assay, which is based on the reduction of a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) tetrazolium compound. Cells were seeded in 96-well plates at a density of 1.2×10^4 cells/well for HeLa and 1.5×10^4 cells/well for MCF10A. After treating cultures for 24 h, assays were performed by adding 10 µL of the CellTiter 96 AQueous One Solution Reagent directly to culture wells, incubating for 15 min (HeLa) or 1 h (MCF10A) at 37 °C, and then recording absorbance on a microplate reader (Infinite M200; Tecan Group Ltd., Männedorf, Switzerland) at both a test wavelength of 490 nm and a reference wavelength of 650 nm to subtract background. All analyses were run in triplicate. The cell viability was calculated as percentage of control values (untreated samples).

4.2.3. Determination of Total Phenolic Content (TPC), Total Flavonoids Content (TFC), Total Chlorophyll and Carotenoids Content

The total phenolic content (TPC) of goji extracts was determined using the Folin–Ciocalteu method as described by Way, M.L. *et al.*, with slight adjustments (Way et al., 2020). Reagent A was prepared by combining 5 mL of 2 M Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) to 45 mL of distilled water. For reagent B, 2.87 g of sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water in a 25 mL volumetric flask. For each sample, 2 µL of extract was added to 100 µL of reagent A in a microplate, mixed, and left for 5 min before adding 70 µL of reagent B and mixing. Then, the microplate was incubated for 1 hour at 40 °C. The absorbance of the solution was then evaluated at 765 nm using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A blank solution was prepared and gallic acid was selected as the standard. Stock solution (1 mg/mL) was prepared in MeOH, and the calibration curve was obtained in a concentration range of 10–500 µg/mL, with six concentration levels (y = 1.779,61x – 2,0811) and the linearity of the standard curve was 99.92%. The solution was measured in triplicate. The total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total flavonoid content was determined applying the method reported by Imeneo, V. *et al.*, appropriately modified (Imeneo et al., 2022). An aliquot of extract (25 µL), 100 µL of deionized water, and 7.5 µL of $NaNO_2$ (5%, w/v) were placed in a microplate and incubated at room temperature (5 min). Then, 7.5 µL of $AlCl_3$ (10%, w/v) were added and incubated for 6 min; after that, 100 µL of NaOH (4%, w/v) was mixed. Simultaneously a blank solution was prepared. The reaction mix was left to settle for 15 min in the dark; after this time, the absorbance was read at 510 nm using a Multiskan

SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and comparing values to rutin calibration standard curve (10–500 μg/mL; y = 2.239,24x – 5,3342; R2 = 99.92%). The results were expressed as milligrams of rutin equivalents per gram of dry weight.

The chlorophyll content was determined using the methods previously described by Wang, H. *et al.* and Zhang, M. *et al.*, and the carotenoids content was determined using the method reported by Samec, D. *et al.*, appropriately modified (Šamec et al., 2014, Wang et al., 2022, Zhang et al., 2022). Briefly, 10 mg of extract was mixed with 1.25 mL of 80% acetone for 5 min on a tube mixer and then centrifuged immediately at 14680 rpm for 10 min. The absorbance of the mixture was measured at 646.8 nm, 663.2 nm and 452.5 nm (Multiskan SkyHigh Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The content of the pigments was calculated by the measured absorbance value, the solution volume, and the sample mass according to the following equations:

$$\text{Chl a (mg/gDW)} = \{[(12.25 \times A\_663.2) - (2.79 \times A\_646.8)] \times V\}/m$$

$$\text{Chl b (mg/gDW)} = \{[(21.50 \times A\_646.8) - (5.10 \times A\_663.2)] \times V\}/m$$

$$\text{Car (mg/gDW)} = \{[(4.75 \times A\_452.5) - (0.226 \times (Chl\ a + Chl\ b))] \times V\}/m$$

where V was the volume of the extract (mL); m was the weight of the sample dry (mg); and A_663.2, A_646.8 and A_452.5 were the absorbance values of the mixture solution at 663.2, 646.8 and 452.5 nm, respectively.

4.2.4. Determination of Antioxidant Activity by DPPH and ABTS Assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay. The DPPH radical scavenging assay was carried out slightly modifying the conditions reported by Noreen, H. and co-workers (Noreen et al., 2017). Briefly, 20 µL of extracts, antioxidant standards, methanol as blank (control) were mixed with 180 µL methanolic solution of DPPH (0.1 mM). Trolox was used as analytical standard (10–100 µg/mL; y = 0,69222x – 2,72353; R2 = 96.13%). All the samples were prepared in triplicate, shaked and incubated in dark for 30 min at 37°C. Changes in the absorbance of the samples were measured against methanol as blank at 517 nm using a microplate reader (Multiskan SkyHigh Microplate Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The result of the DPPH reduction in the sample was reported both as mol Trolox equivalents per gram of dry weight (mol TE/g DW) as percentage DPPH inhibition calculated using the formula:

$$DPPH\ inhibition\ (\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

The ABTS test was performed as described by Walker and Everette (Walker and Everette, 2009) with modifications. The ABTS solution was prepared with 38.4 mg of ABTS to which was added 6.62 mg of potassium persulphate ($K_2S_2O_8$) and finally 10 mL of MilliQ water. The mixture was kept in the dark for 16 hours. The ABTS solution was diluted 50-fold with PBS buffer to obtain an optimal absorbance ranging from 1.20 to 1.30 at 734 nm (A734) using a conventional 1 cm spectrophotometer. Subsequently, 2.5 µL of samples or reference compound (Trolox), previously prepared with 5 mg of Trolox solubilized in 10 mL of PBS, were added to the 96-well microplate followed by 250 µL of ABTS solution. The plates were incubated in the dark for 20 min at room temperature

and A734 was recorded using a microplate reader (Infinite M200; Tecan Group Ltd., Männedorf, Switzerland). The result of the ABTS assay was reported both as mol Trolox equivalents per gram of dry weight (mol TE/g DW) as percentage ABTS scavenging.

4.2.5. Metal Binding Studies

The metal binding studies were performed as described (Umar et al., 2018). The UV absorption of the LBE (500 µg/mL) alone or in the presence of $CuSO_4$, $FeSO_4$ or $FeCl_3$ (40 µM) for 30 min in 20% (v/v) ethanol/ buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) was recorded using a microplate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA) with wavelength ranging from 280 to 400 nm. The final volume of reaction mixture was 1 mL.

4.2.6. Lycium barbarum Extract LCMS Identification: UHPLC-HRMS/MS

UHPLC-HRMS/MS analysis was performed on a Thermo Ultimate RS 3000 coupled online to a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionization probe (HESI II).

The separation was performed in reversed phase mode, with a Kinetex® 2.6µm EVO C18 100 Å, LC Column 50 x 2.1 mm (Phenomenex, Bologna, Italy) with a guard cartridge system (SecurityGuard ULTRA cartridges for EVO-C18, sub-2µm and core-shell columns with 2.1mm internal diameters). The column temperature was set at 40°C and the flow rate was 0.4 mL/min. The mobile phase was (A): H_2O with 0.1 % HCOOH (v/v) and (B): ACN with 0.1 % HCOOH (v/v). The following gradient was employed: 0.01–3.00 min, isocratic to 0% B; 3.01–10.00 min, 0-10% B; 10.01–25.00 min, 10-20% B; 25.01-30.00 min, 20-50% B; 30.01-33.00 min, 50-95% B; 33.01–36.00 min, isocratic to

95% B; 36.01–38.00 min, 95-0%; then six minutes for column re-equilibration. Injection volume was 5 µL. All additives and mobile phases were LCMS grade and purchased from Merck (Milan, Italy).

The ESI was operated in positive and negative mode. The MS was calibrated by Thermo CalMix Pierce™ calibration solutions in both polarities. Full MS (100-1500 m/z) and data-dependent MS/MS were performed at a resolution of 35,000 and 17,500 FWHM respectively, normalized collision energy (NCE) values of 15, 20, and 25 were used. Source parameters: Sheath gas pressure, 50 arbitrary units; auxiliary gas flow, 13 arbitrary units; spray voltage, +3.5 kV, -2.8 kV; capillary temperature, 310°C; auxiliary gas heater temperature, 300°C.

The identification of investigated analytes was carried out by comparing their retention times and MS/MS data with those present in the literature. The MS spectra were processed using FreeStyle™ 1.8 SP2 and the commercial software Compound Discoverer v. 3.3.1.111 SP1 (Thermo Fisher, Bremen, Germany), and identification was accomplished by activating the Chem Spider and mzCloud nodes. The following online databases were also consulted: Phenol-Explorer (www.phenolexplorer.eu), PubChem (https://pubchem.ncbi.nlm.nih.gov), SciFinder Scholar (https://scifinder.cas.org).

4.2.7. Cell Cultures and LBE Treatment

The human breast cancer MCF-7 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/mL glucose) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Cells were routinely grown in culture dishes (Corning, Corning, NY) in an environment containing 5% CO_2 at 37 °C and splitted every 2 days. In each experiment,

cells were placed in a fresh medium and cultured in the presence of the LBE at different concentrations and times. Each treatment and analysis were performed at least in triplicate separate experiments.

4.2.8. Cell Viability Assay on Human Breast Cancer MCF-7 Cell Line

Cell viability was established by measuring mitochondrial metabolic activity with 3-[4,5-dimethylthiazol-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Quagliariello et al., 2022). Briefly, MCF-7 (6×10^3 cells/well) and MCF-10A (6×10^3 cells/well) were plated into 96-well plates, then the LBE (3–100 µg/mL) was added for 24 h. Afterward, MTT reagent at 0.5 mg/mL final concentration for 2 h was added. Then, 100 µL per well of 0.1 M isopropanol/HCl solution was added to dissolve the formazan crystals. The absorbance was measured at 570 nm, using a microplate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA). Cell viability was expressed as a percentage relative to the untreated cells cultured in medium with 0.1% DMSO and set to 100%, whereas 10% DMSO was used as positive control and set to 0% of viability. The EC_{50} values were calculated using GraphPad Prism 8.0 software by nonlinear regression of the dose-response inhibition.

4.2.9. Determination of Hypodiploid Nuclei

Hypodiploid nuclei were analyzed using propidium iodide (PI) staining by flow cytometry as described previously. MCF-7 cells (4×10^4 cells/well) were grown in 12-well plates and allowed to adhere for 24 h. Later the medium was replaced, and cells were treated with LBE (25, 12, 6 µg/mL) for 24 h. After treatments, the culture medium was replaced, cells washed twice with PBS and then suspended in 300 µL of a hypotonic staining solution containing 50 µg/mL PI, 0.1% (w/v) sodium citrate, and 0.1% Triton X-

100. Culture medium and PBS were centrifuged, and cell pellets were pooled with cell suspension to retain both dead and living cells for analysis. After incubation at 4 °C for 30 min in the dark, cell nuclei were analyzed with a Becton Dickinson FACScan flow cytometer using the Cell Quest software, version 4 (Franklin Lakes, NJ, USA). Cellular debris was excluded from the analysis by raising the forward scatter threshold, then the percentage of cells in the hypodiploid region (sub G0/G1) was calculated.

4.2.10. PI/Hoechst 33342 Double Staining Assay

Cell-permeable DNA dye Hoechst 33342 (Di Micco et al., 2022) and PI were used to validate necrosis in cell populations. MCF-7 cells (4×10^4 cells/well) seeded on glass cover slips were grown in 12-well plates and allowed to adhere for 24 h. Next the medium was replaced, and cells were treated with LBE (25, 12, 6 µg/mL) for 24 h. After treatments the culture medium was replaced, and live cells were stained with a Hoechst 33342/PI solution added to cell culture medium (Hoechst 33342, 5 µM; PI, 30 µg/mL) at 37 °C for 20 min in the dark. The stained cells were washed two times and then fixed with 3.7 % formaldehyde for 10 min. Images were acquired on a fluorescence microscope (Axioshop 40, Zeiss. Magnification, 20×). Quantitative analyses were performed by the ImageJ program, version 1.47 ($N \geq 10$).

4.2.11. ROS Detection

ROS levels were measured using 10 µM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma Aldrich, St. Louis, MO, USA). To test the effect of LBE (50, 25, 12 and 6 µg/mL) to ROS neutralization, MCF-7 cells were seeded (4×10^4 cells/well) in 12-well plates allowing to adhere for 24 h. Next, cells were incubated for 1-24 h with LBE. After treatments, the medium was removed, and the cells were washed

twice with PBS. A staining solution containing DCFH-DA in serum-free medium without phenol-red was added for 20 min at 37 °C in the dark. The fluorescence signals were evaluated using Becton Dickinson FACScan flow cytometer and analyzed with Cell Quest software, version 4 (Franklin Lakes, NJ, USA).

4.2.12. RNA Isolation and Absolute Quantification of Nox1-5 Gene Expression by ddPCR

The MCF-7 and MCF10A cells, after being incubated for 24 hours at 37 °C in a humidified atmosphere of 5% (v/v) CO_2, were treated for 24 hours with the extract of *L. barbarum* at a concentration of 100 µg/mL.

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Samples were treated with RNase-free DNase (Qiagen GmbH) to prevent amplification of genomic DNA.

The reverse transcription phase consists in the transcription of messenger RNA molecules for the production of cDNA molecules, by the viral enzyme "Reverse Transcriptase". Aliquots of about 1 µg of total RNA were subjected to reverse transcription for 1h at 37 °C using the "Ready-to go You-Primer First-Strand Beads" kit (code 27-9264-01 Amersharm Pharmacia biotech), containing both the enzyme and the nucleotides in a freeze-dried bead, in a reaction mixture to which 0.5 µg of Oligo-dT primer are added.

Absolute quantification of RNA levels was performed by microfluidic droplet digital PCR (ddPCR). The ddPCR procedure was conducted using the QIAcuity 2-plex instrument (Qiagen, Hilden, Germany). The ddPCR reaction mix was developed using QIAcuity 3X Eva Green PCR Master Mix, 10X specific primer sets (4M) (Qiagen, Hilden, Germany), RNase-free water and a specific concentration of cDNA (100 ng),

arriving at a final volume of 15 μL per sample. After accurate vortexing, then the samples were loaded into 24-well 8.5K (8500 partitions) nanoplates and sealed with the nanoplate seal. The primers used were listed as below: NOX1 forward primer: 5'-GTT TTA CCG CTC CCA GCA GAA-3′, reverse primer: 5'-GGA TGC CAT TCC AGG AGA GAG-3′; NOX2 forward primer: 5'-GGA GGA TCC GTG GTC ACT CAC CCT TT CAA-3′, reverse primer: 5'-CCA CTC GAG CTC ATG GAA GAG ACA AGT TAG-3′; NOX3 forward primer: 5'-GCA GGA TCC GTG GTA AGC CAC CCC TCT G-3′, reverse primer: 5'-GCT GAA TTC AGA AGC TCT CCT TGT TGT AAT-3′; NOX4 forward primer: 5'-GCA GGA TCC GTC ATA AGT CAT CCC TCA GA-3′, reverse primer: 5'-GCT GTT AAC GTC GAC TCA GCT GAA AGA CTC TTT AT-3′; NOX5 forward primer: 5′-GGA GGA TGC CAG GTG GCT CCG GT-3′, reverse primer: 5'-AGC CCC ACT ACC ACG TAG CCC-3′ (Dou et al., 2019).

The 8.5K nanoplate gives rise to 8500 individual partitions where each sample is randomly distributed. The QIAcuity carries out fully automated processing including all necessary steps for plate priming, sealing of partitions, thermocycling, and image analysis. The amplification protocol provides for a first step at 95 °C for 2 min necessary for enzyme activation involved in the amplification. Subsequently 40 cycles in which each single cycle includes the following steps: 15 s at 95 °C for denaturation; 15 s at 60 °C for annealing; 15 s at 72 °C for extension; 5 min at 40 °C final step to complete any remaining incomplete fragments.

Fluorescent light is emitted from positive partitions that have a target molecule, compared to negative partitions that have no target molecules. The experiments were performed using a negative control without cDNA. Data were analyzed using QIAcuity Suite V1.1.3 software (Qiagen) and results are expressed in cDNA copies/μL based on Poisson statistical analysis.

4.2.13. Western Blotting Analysis

MCF-7 cell line was seeded in 60 mm culture dishes, treated with LBE (6 μg/mL) for different times (2 h, 4 h, 8 h). After 8 h, cells were washed twice with PBS and detached with a scraper, centrifuged at 655g × 10 min at 4 °C. Full proteins were extracted by lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 2% NP-40, 1% sodium deoxycholate, 1× protease and phosphatase inhibitor cocktail) for 30 min. Then, cell lysates were centrifuged at 4850g for 20 min at 4 °C. 30 μg of total proteins were run on 8–12% SDS-PAGE and transferred to nitrocellulose membranes using a minigel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Blots were blocked in phosphate buffered saline, containing Tween-20 0.1% and 10% non-fat dry milk for 1 h at room temperature and incubated overnight with specific primary antibodies at 4 °C with slight agitation. α-tubulin was used as the loading control. The following antibodies were used: rabbit monoclonal anti-PERK (Cell Signaling, Danvers, MA, USA), mouse monoclonal anti-ATF6 (Cell Signaling, Danvers, MA, USA), rabbit polyclonal anti-caspase-12 (Abcam, Cambridge, UK), mouse monoclonal anti-α-tubulin (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-phospho-IRE1α (Sigma Aldrich, St. Louis, MO, USA), mouse monoclonal anti-NRLP3 (Sigma Aldrich, St. Louis, MO, USA), rabbit polyclonal anti-GRP78 (Sigma Aldrich, St. Louis, MO, USA), rabbit monoclonal anti-Nrf2 (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal anti-CHOP (Sigma Aldrich, St. Louis, MO, USA). After washes in PBS/Tween-20 0.1%, the appropriate anti-rabbit or anti-mouse (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) peroxidase-linked secondary antibody was added for 1 h at room temperature. Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL kit, Amersham, Germany). Filters were exposed to LAS 4000

(GE Healthcare, Chicago, IL, USA) and the densitometry analysis of autoradiographs was performed by the ImageJ program, version 1.47.

4.2.14. RNA Extraction and Reverse-Transcription and Real-time PCR

Total RNA was isolated from treated cells using Trizol reagent (Gibco, Thermo Fisher Scientific, MA, USA), according to manufacturer's instructions. Concentration and purity of RNA were determined spectrophotometrically by reading the absorbance of ratios 260/280 and 260/230 nm. Aliquots of total RNA for Real-time PCR test were subjected to DNase I digestion (Thermo Fisher Scientific, MA, USA) and reverse transcribed using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocol. Thermal conditions for reverse transcription were 25 °C for 10 min, 37 °C for 50 min, and 75 °C for 15 min. In the last step, RNAse H was added (Vestuto et al., 2022).

Real-time PCR was performed with QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific, MA, USA) using SYBR Green detection in a total volume of 20 µL with 1 µL of forward and reverse primers (5 µM) and 10 µL of PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, MA, USA). Reactions included an initial cycle at 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, extension at 72 °C for 1 min. Values were determined from standard curve generated from serial cDNA dilutions and normalized to GAPDH.

The primers used for the qPCR reactions are the following: forward ATF4 5′-GTC CCT CCA ACA ACA GCA AG-3′; reverse ATF4 5′-CTA TAC CCA ACA GGG CAT CC-3′; forward Xbp-1 5′-TTA CGA GAG AAA ACT CAT GGC-3′; reverse Xbp-1 5′-GGG TCC AAG TTG TCC AGA ATG C-3′. The 2−ΔΔCT method was used to analyze

the results and relative mRNA expression levels were determined as fold-induction relative to Ctrl cells, set as 1.

4.2.15. Statistical Analysis

Data are reported as mean ± SD of results from three independent experiments. Statistical analysis was performed using an analysis of variance test (ANOVA), and multiple comparisons were made with the Bonferroni's test with GraphPad Prism 8.0 software (San Diego, CA, USA). Significance was assumed at $p < 0.05$.

4.3. Results

4.3.1. Screening to Evaluate Cytotoxic Activity of the Extracts on Tumor and Non-Tumor Cells

In order to evaluate the potential cytotoxic effect, the ethanolic extracts of *Malpighia emarginata* (MME), *Arbutus unedo* (AUE), *Lycium barbarum* L. (LBE), *Annona cherimola* (ACE), *Diospyros kaki* (DKE) were initially tested on a cervical cancer tumor cell line HeLa. The cell line was treated with increasing concentrations (between 100 and 2000 µg/mL for MEE and between 25 and 500 µg/mL for each other extract) for 24 hours. The data showed that all extracts had cytotoxicity against the cervical cancer cell line HeLa (Figure 44). Among the extracts, LBE showed higher antiproliferative effects than the other extracts. Notably, the cytotoxicity of LBE with IC_{50} values of 191,5 µg/mL was much lower than the values found for the other extracts in HeLa cells. In fact, MEE, AUE, DKE and ACE extracts showed low cytotoxic potential with IC_{50} values > 300 µg/mL up to IC_{50} > 1000 µg/mL for MEE. Data showed that the extracts exhibit a dose-dependent effect on cell viability of HeLa cell line, with the greatest reduction obtained from Goji berries extract.

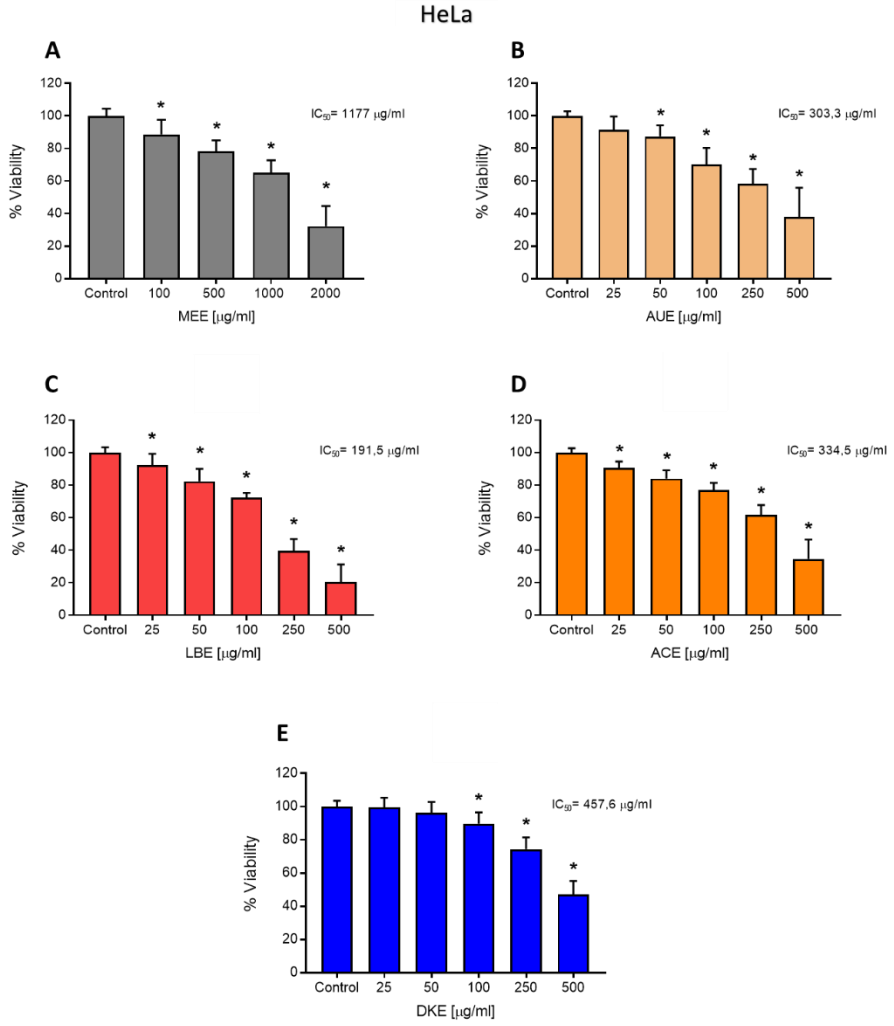


Figure 44. *Dose-response curves of MEE (**A**), AUE (**B**), LBE (**C**), ACE (**D**), DKE (**E**) extracts on cell viability. HeLa cells were treated for 24 hours with increasing concentrations between 100 and 2000 µg/mL for MEE and between 25 and 500 µg/mL for the other extracts. Goji Berry extract showed the highest cytotoxicity with an IC_{50} of 191,5 µg/mL on HeLa cell. Values are presented as the mean ± SD from 5 separate experiments and expressed as a percentage of control values. * P <0.05 compared to control values.*

Subsequently all the extracts were tested on a healthy cell line, in order to evaluate their possible impact on viability. In particular, the non-tumor breast cell line MCF10A was used. The cell line was then treated with the same increasing concentrations used for tumor cells (between 100 and 2000 µg/mL for MEE and between 25 and 500 µg/mL for

each other extract) for 24 hours. At the end of the experiments, only slight effects on cell viability were observed (Figure 45), supporting the absence of significant toxicity of the studied extracts (IC_{50} >>1000 µg/mL), with the exception of ACE which presents, at the maximum concentration tested (500 µg/mL), a toxic effect on healthy cells.

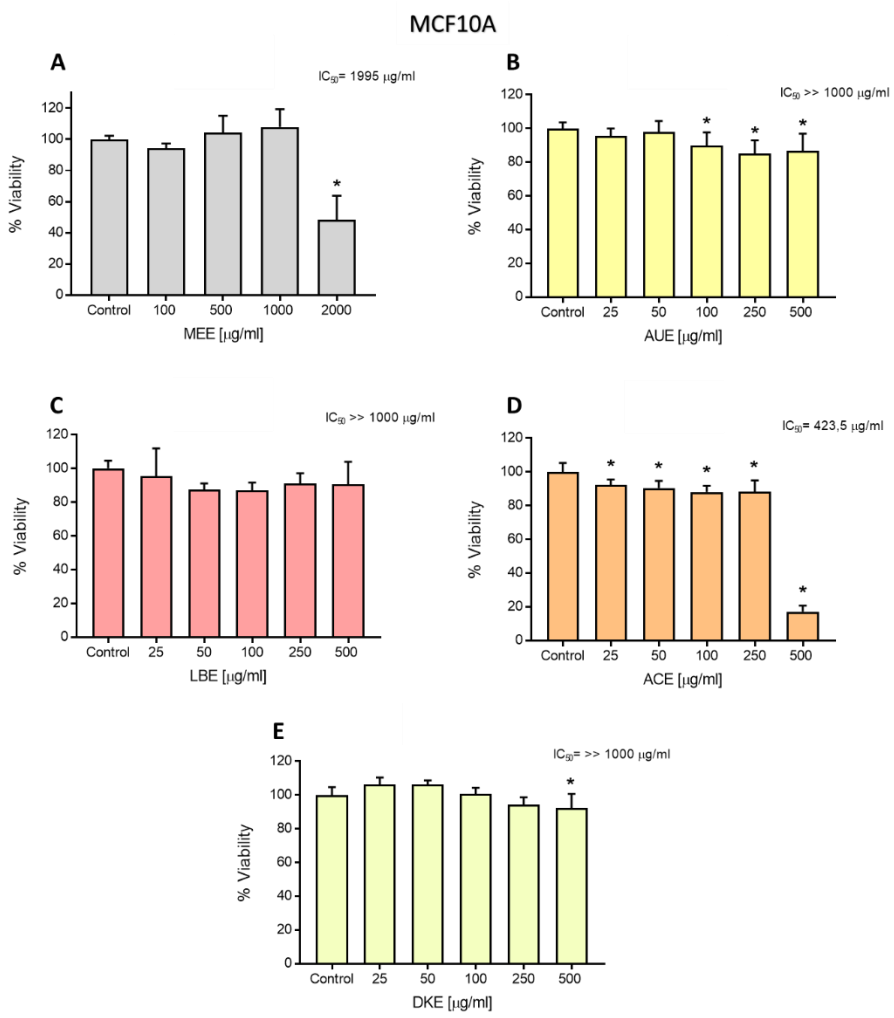


Figure 45. *Dose-response curves of MEE (**A**), AUE (**B**), LBE (**C**), ACE (**D**), DKE (**E**) extracts on cell viability. MCF10A cells (non-malignant breast epithelial cells) were treated for 24 hours with increasing concentrations between 100 and 2000 µg/mL for MEE and between 25 and 500 µg/mL for the other extracts. The extracts have an IC_{50} much higher than 1000 µg/mL, with exception of ACE effect which presents a toxic effect on healthy cells (highest tested concentration, 500 µg/mL). Values are presented as the mean ± SD from 5 separate experiments and expressed as a percentage of control values. * P <0.05 compared to control values.*

The results obtained suggest a potential use of the tested extracts in the onconutraceutical field, in particular for the ethanolic extract of *L. barbarum* which has the best overall profile on both tumor and healthy cell lines (Figure 46).

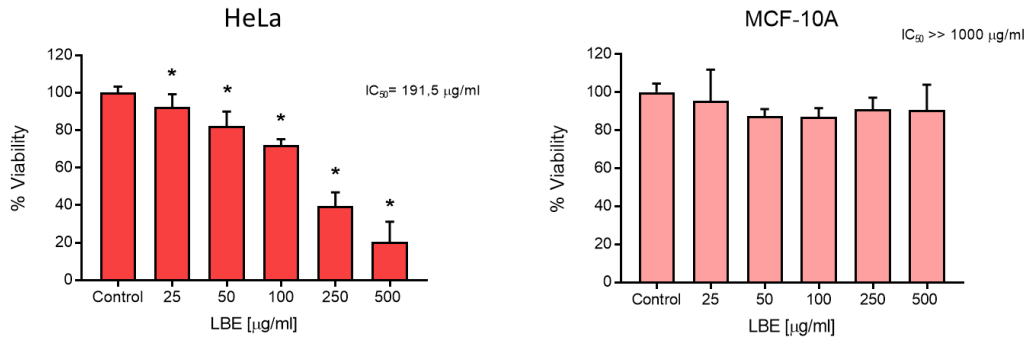


Figure 46. *Effects of L. barbarum extract on tumor (HeLa) and non-tumor (MCF10A) cell lines. Values are presented as the mean ± SD from 5 separate experiments and expressed as a percentage of control values.* ***** *P <0.05 compared to control values.*

Data analysis from this initial screening showed that *L. barbarum* extract had the highest antiproliferative capacity on the HeLa cervical cancer cell line and had no significant effect on MCF10A healthy cells. Subsequently, since the IC_{50} on HeLa cells was slightly elevated and since MCF10A cells were used as healthy cells, it was thought to use the corresponding breast cancer cell line MCF-7 to evaluate the possible biological properties of the LBE extract.

4.3.2. Total Phenolic, Flavonoid, Chlorophyll and Carotenoid Content, and Antioxidant Activity

In order to evaluate the biological properties of the *L. barbarum* extract, several analyzes were performed to clarify the phenolic, flavonoid, chlorophyll and carotenoid content. In particular, the Folin-Ciocalteu method, a recognized protocol for the

quantitative determination of total phenolic compounds, was used to evaluate the total phenolic content (TPC). In general, the LBE extract showed good TPC values. The total phenolic content was calculated and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW). The amount of TPC was 31.26 ± 0.01 mg GAE/g DW (Table 7).

Subsequently, the total flavonoid content (TFC) was evaluated. The TFC was evaluated using a protocol present in the literature with some modifications (Imeneo et al., 2022). The LBE extract showed a good content of total flavonoids. The results were expressed as milligrams of rutin equivalents (RE) per gram of dry weight (DW) resulting in 65.51 ± 0.01 mg RE/g DW (Table 7).

To evaluate the possible biological properties of the ethanolic extract of *L. barbarum*, the content of chlorophylls and carotenoids was also evaluated. From the data obtained it emerges that the content of chlorophyll a (Chl a), expressed in mg/g of dry weight, is 0.417 ± 0.001 mg/g DW, the content of chlorophyll b (Chl b) is 0.693 ± 0.019 mg/ g DW, while the total chlorophyll content (Chl a + Chl b) is 1.131 ± 0.001 mg/g DW. The total content of carotenoids present in the ethanolic extract was also evaluated and was found to be 0.508 ± 0.004 mg/g DW (Table 7).

The antioxidant activity of the extract was evaluated by the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals and by ABTS assay. The result of the DPPH reduction in the sample was reported both as mol Trolox equivalents (TE) per gram of dry weight (mol TE/g DW) as percentage DPPH inhibition. The scavenging activity of DPPH radicals of LBE is 154.85 ± 0.03 mol TE/g DW and the inhibition percentage was 59.96 ± 0.01. Similarly, the results of the ABTS assay were reported both as mol Trolox equivalents per gram of dry weight (mol TE/g DW) as percentage ABTS scavenging. LBE had an ABTS scavenging activity of 115.5 ± 0.04 mol TE/g DW and a scavenging

percentage of 25.5 ± 0.02 (Table 7). Table 7 show the phenolic, flavonoid, chlorophyll and carotenoid content and DPPH and ABTS reduction.

Table 7. *Phenolic, flavonoid, chlorophyll and carotenoid content and antioxidant capacity.*

Assay	LBE	Measure Unit
TPC	31.26 ± 0.01	mg GAE/g DW
TFC	65.51 ± 0.01	mg RE/g DW
Chl a	0.417 ± 0.001	mg/g DW
Chl b	0.693 ± 0.019	mg/g DW
Chl a + Chl b	1.131 ± 0.001	mg/g DW
Carotenoid	0.508 ± 0.004	mg/g DW
DPPH	154.85 ± 0.03	mg TE/g DW
	59.96 ± 0.01	% inhibition
ABTS	115.5 ± 0.04	mg TE/g DW
	25.5 ± 0.02	% inhibition

Data are expressed as the mean of triplicate analyses ± standard deviation

Metal binding studies were performed to further support these results. The results show that LBE also exerts its antioxidant activity through chelation with iron and copper ions (Figure 47), in agreement with the high polyphenolic content and DPPH and ABTS scavenging action determined with previous assays.

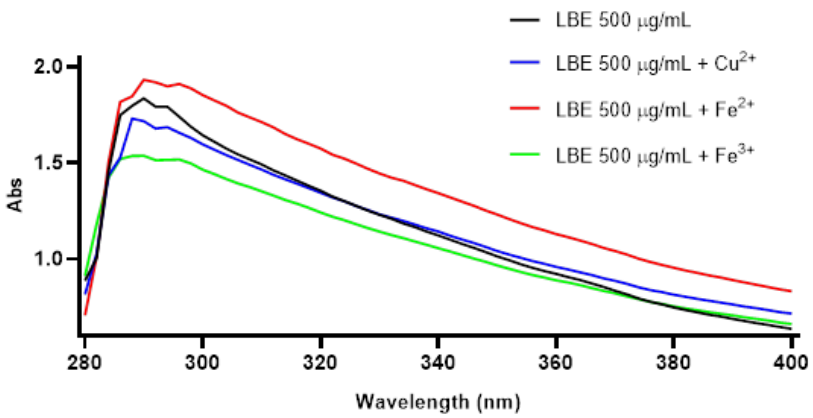


Figure 47. *UV spectra (in range 280 to 400 nm) of LBE (500 µg/mL) alone and in the presence of 40 µM $FeSO_4$, $FeCl_3$ and $CuSO_4$.*

4.3.3. Polyphenolic Profile of Lycium barbarum Extract

The study involved the analytical characterization of the main polyphenolic molecules contained in the ethanolic extract isolated from the *L. barbarum*. Figure 48 shows the Total Ion Chromatogram (TIC) profiles, in negative ionization mode, of polyphenolic compounds isolated from *Lycium barbarum*.

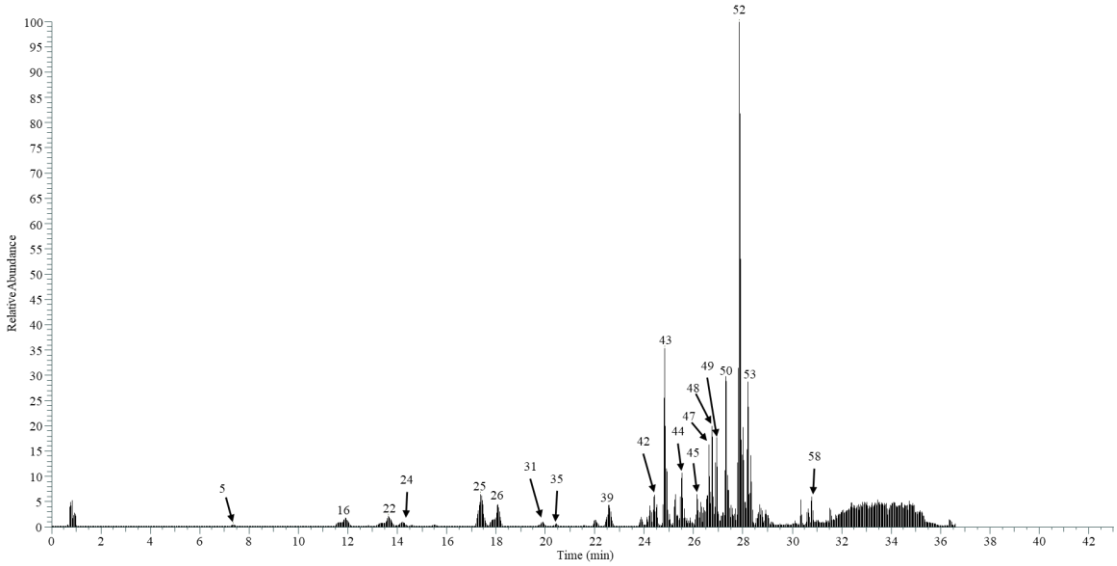


Figure 48. *Representative total ion chromatogram in negative ionization mode.*

The LC−MS/MS analyses allowed us to identify 60 compounds in the investigated goji sample (Table 8), in negative and positive ionization mode. Among them, were present some classes of free polyphenols, such as phenolic acids, phenylpropanoids and flavonoids. Table 8 reports all tentatively identified compounds with their chromatographic retention times, MSn fragmentation ions and assigned identities.

In the goji extract, the hydroxy fatty acids were identified. Chromatographic peaks **52** exhibited the precursor ion at m/z 295, but the loss of a water molecule $[M-H-18]^-$ and the relative cleavage of the C = C bond at C9–C10 produced fragments at *m/z* 277 and

171, leading to its tentative identification as hydroxy octadecadienoic acid (HODE) (Oliw et al., 1998).

Moreover, peaks **47**, **48** and **49** were observed at different retention times but all gave $[M–H]^-$ ions at *m/z* 311 and an MS/MS fragmentation pattern characterized by a base peak at *m/z* 293, deriving from the loss of a water molecule $[M–H–18]^-$. Thus, these compounds were tentatively identified as identified dihydroxy octadecadienoic acid (DiHODE) (Oliw et al., 1998). As reported literature showed, all hydroxy fatty acids were characterized by 18 carbon atoms and their fragmentation pattern showed losses of water molecules and aliphatic residues. In addition, also trihydroxy octadecadienoic acid (TriHODE) were identified (peaks **42**, **43** and **45**) (Pontieri et al., 2021, Kang et al., 2016).

Many flavonoids have been found in LBE. Compound **21** were tentatively identified as Rutin hexoside giving *m/z* 771 $[M–H]^-$, whilst an MS/MS fragmentation pattern characterized by *m/z* 609 $[M–H–162]^-$ deriving from the loss of sugar moiety and corresponding to the Rutin aglycone, also identified as compound **26** (Bondia-Pons et al., 2014, Lu et al., 2014).

UHPLC-ESI positive analysis (Figure 49) highlighted the presence of compound **57**, tentatively identifies as oleamide, showing a precursor ion $[M–H]^+$ at *m/z* 282 and a base fragment peak at *m/z* 265 and *m/z* 247 due to loss of ammonia (17 Da) and both ammonia and water (35 Da) from the carboxamide (Divito et al., 2012).

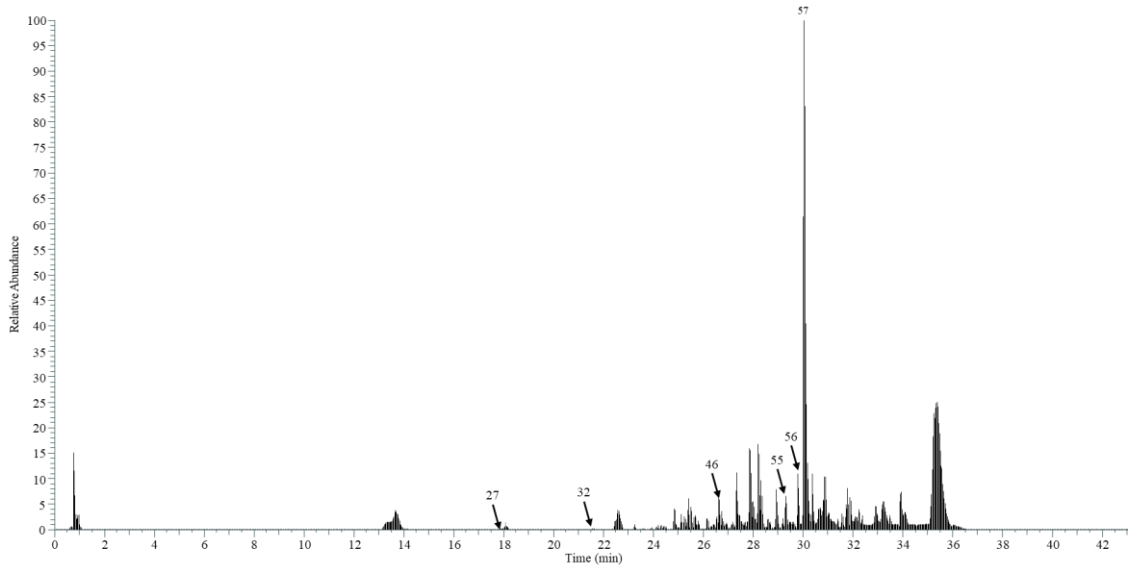


Figure 49. *Representative total ion chromatogram in positive ionization mode.*

Table 8. *Identified compounds of extract from Lycium barbarum.*

Peak	Compounds	Rt (min)	$[M-H]^-$	$[M+H]^+$	MS/MS	Molecular formula	Error (ppm)	Classification	Reference
1	Gallic acid	2.37	169.0136	-	125.0233	$C_7H_5O_5$	1.55	Phenolic acids	(Liu et al., 2021)
2	p-Coumaric acid-glycosides isomer (I)	3.41	325.0934	-	119.0491 163.0392	$C_{15}H_{18}O_8$	0.76	Phenylpropanoids	(Liu et al., 2021)
3	Hydroxybenzoic acid	5.15	137.0235	-	93.0333	$C_7H_6O_3$	0.74	Phenolic acids	(Liu et al., 2021)
4	Benzoic acid	6.68	121.0285	-	/	$C_7H_6O_2$	-0.33	Organic acids	(Liu et al., 2021)
5	4-(2-Carboxyethenyl)-2-(3,4-di hydroxy phenyl)-2,3-dihydro-7-hydroxy-3-methylester, [2α, 3β, 4(E)-3-benzofuran carboxylic acid	7.37	371.0988	-	119.0491 163.0392	$C_{16}H_{20}O_3$	2.69	Flavonoids	(Duan et al., 2022)
6	3,4-di-O-caffeoylquinic acid	8.60	515.1413	-	353.0885 323.0759	$C_{22}H_{28}O_{14}$	1.77	Phenylpropanoids	(Liu et al., 2021)
7	Caffeic acid	9.06	179.0344	-	135.0441	$C_9H_8O_4$	1.13	Phenylpropanoids	(Liu et al., 2021)
8	p-Coumaric acid-glycosides isomer (II)	9.35	325.0934	-	145.0285 163.0391	$C_{15}H_{18}O_8$	4.42	Phenylpropanoids	(Liu et al., 2021)
9	3,5-di-O-caffeoylquinic acid	10.10	515.1415	-	191.0554 179.0333	$C_{22}H_{28}O_{14}$	3.78	Phenylpropanoids	(Liu et al., 2021)
10	*N*-Caffeoyl, *N'*-dihydrocaffeoyl spermidine dihexose	10.46	-	796.3496	163.0388 220.0966 310.2125 472.2498	$C_{37}H_{53}O_{16}N_3$	-1.31	Alkaloids	(Wang et al., 2018, Yossa Nzeuwa et al., 2017)
11	N^1-Dihydrocaffeoyl, N^{10}-caffeoyl spermidine hexose	11.25	-	634.2977	163.0389 222.0967 165.0546 472.2523 310.2133	$C_{31}H_{44}O_{11}N_3$	-0.54	Alkaloids	(Wang et al., 2018, Yossa Nzeuwa et al., 2017)
12	Ferulic acid hexoside	11.26	355.1041	-	175.0392 160.0157	$C_{16}H_{20}O_9$	6.70	Phenylpropanoids	(Bondia-Pons et al., 2014)

13	p-Coumaric acid-glycosides isomer (III)	11.40	325.0933	-	145.0285 163.0390 119.0491	$C_{15}H_{18}O_8$	2.26	Phenylpropanoids	(Liu et al., 2021)
14	*N,N'*-bis-(caffeoyl)-spermidine isomer	11.76	470.2301	-	135.0442	$C_{25}H_{32}O_6N_3$	-4.32	Phenolamides	(Yossa Nzeuwa et al., 2017)
15	N^1-Dihydrocaffeoyl, N^{10}-caffeoyl spermidine	11.77	-	472.2445	163.0389 220.0967 310.2130 293.1865 236.1276	$C_{25}H_{34}O_6N_3$	1.08	Alkaloids	(Wang et al., 2018, Yossa Nzeuwa et al., 2017)
16	p-Coumaric acid	11.88	163.0392	-	119.0490	$C_9H_8O_3$	0.94	Hydroxycinnamic acids	(Liu et al., 2021)
17	p-Coumaric acid	11.97	-	165.0547	147.0439 119.0492	$C_9H_8O_3$	-0.96	Hydroxycinnamic acids	(Yang et al., 2020)
18	N^1,N^{10}-bis-(Caffeoyl) spermidine dihexose	12.07	-	794.3350	163.0389 220.0969 308.1956	$C_{40}H_{49}O_{13}N_4$	-0.28	Alkaloids	(Wang et al., 2018, Yossa Nzeuwa et al., 2017)
19	p-Coumaroyl-quinic acid	12.76	337.0935	-	191.0553 93.0333	$C_{16}H_{18}O_8$	3.09	Phenylpropanoids	(Liu et al., 2021)
20	6-O-*trans*-feruloyl-2-O-β-d-glucopyranosyl-α-d-glucopyranoside	12.80	517.1569	-	193.0500 175.0392	$C_{22}H_{30}O_{14}$	2.32	Phenylpropanoids	(Liu et al., 2021)
21	Rutin hexose	13.35	771.2005	-	609.1435 462.0828 301.0348	$C_{33}H_{40}O_{21}$	2.72	Flavonoids	(Bondia-Pons et al., 2014)
22	Scopoletin	13.70	191.0343	-	176.0107 148.0156	$C_{10}H_7O_4$	2.18	Coumarins	(Liu et al., 2021)
23	Ferulic acid	14.11	193.0501	-	134.0363 178.0264 149.0598	$C_{10}H_{10}O_4$	2.42	Phenylpropanoids	(Liu et al., 2021)
24	N-acetyl-DL-tryptophan	14.23	245.0931	-	203.0820 74.0235 116.0493	$C_{13}H_{14}O_3N_2$	3.93	Tryptophan derivate	(Bondia-Pons et al., 2014)
25	Azelaic acid	17.44	187.0968	-	125.0961 97.0646	$C_9H_{16}O_4$	2.02	Fatty acid	(Bondia-Pons et al., 2014)
26	Rutin	18.05	609.1463	-	300.0275 301.0356	$C_{27}H_{30}O_{16}$	0.41	Flavonoids	(Liu et al., 2021)
27	Rutin	18.07	-	611.1603	303.0497 465.1002	$C_{27}H_{30}O_{16}$	-0.63	Flavonoids	(Yang et al., 2020)
28	Quercetin-3-O-galactoside	18.24	463.0886	-	300.0275 301.0358	$C_{21}H_{20}O_{12}$	2.69	Flavonoids	(Liu et al., 2021)
29	N-p-*cis*-coumaroyl-tyramine	18.81	282.1139	-	119.0491 243.0793	$C_{17}H_{17}O_3N$	4.28	Phenylpropanoids	(Liu et al., 2021)
30	Prunin (Naringenin-7-O-glucoside)	18.99	433.1145	-	271.0610	$C_{21}H_{22}O_{10}$	2.38	Flavonoids	(Liu et al., 2021)
31	Nicotiflorin (Kaempferol-3-O-Glu-7-O-Rha)	19.90	593.1516	-	285.0405 255.0298	$C_{27}H_{30}O_{15}$	2.04	Flavonoids	(Liu et al., 2021)
32	Kaempferol-3-O-rutinoside	19.96	-	595.1658	287.0550	$C_{27}H_{30}O_{15}$	-0.37	Flavonoids	(Yang et al., 2020)
33	N-*cis*-feruloyltyramine	20.05	312.1245	-	148.0520 297.1012	$C_{18}H_{19}O_4N$	3.75	Phenylpropanoids	(Liu et al., 2021)
34	Kaempferol-3-O-Glucoside	20.06	447.0939	-	284.0340 285.0403 255.0299	$C_{21}H_{20}O_{11}$	-0.87	Flavonoids	(Liu et al., 2021)
35	Isorhamnetin-3-O-rutinoside	20.45	623.1626	-	315.0511 299.0198	$C_{28}H_{32}O_{16}$	2.13	Flavonoids	(Liu et al., 2021)
36	Isorhamnetin-3-O-galactoside	20.68	477.1046	-	314.0432 243.0302 271.0262	$C_{22}H_{22}O_{12}$	1.09	Flavonoids	(Liu et al., 2021)
37	N-p-*trans*-coumaroyltyramine	21.60	282.1139	-	119.0491	$C_{17}H_{17}O_3N$	4.28	Phenylpropanoids	(Liu et al., 2021)
38	N-p-*trans*-coumaroyltyramine	21.63	-	284.1280	147.0439 121.0649 164.0692	$C_{17}H_{17}O_3N$	-0.88	Phenylpropanoids	(Liu et al., 2021)
39	N-*trans*-feruloyltyramine	22.62	312.1245	-	148.0520 297.1007	$C_{18}H_{19}O_4N$	4.15	Phenylpropanoids	(Liu et al., 2021)
40	Quercetin	23.36	301.2023	-	151.0028 178.9983	$C_{15}H_{10}O_7$	4.13	Flavonoids	(Liu et al., 2021)

41	Naringenin	23.82	271.0615	-	119.0491 151.0028	$C_{15}H_{12}O_5$	5.03	Flavonoids	(Liu et al., 2021)
42	9,12,13-trihydroxy-10,15-octadecadienoic acid	24.46	327.2179	-	171.1019 211.1336 291.2002	$C_{18}H_{32}O_5$	4.33	Fatty acid (TriHODE)	CD
43	9,10,11-trihydroxy-(12z)-12-octadecenoic acid	24.84	329.2334	-	171.1018 139.1117 211.1336	$C_{18}H_{34}O_5$	3.76	Fatty acid	(Lu et al., 2014) CD
44	9,12,13-trihydroxy-10,15-octadecadienoic acid	25.48	327.2180	-	201.1126 213.1127 291.1967	$C_{18}H_{32}O_5$	3.68	Fatty acid (TriHODE)	CD
45	9,12,13-trihydroxy-10,15-octadecadienoic acid	26.11	327.2180	-	171.1019 201.1127 291.1961	$C_{18}H_{32}O_5$	4.05	Fatty acid (TriHODE)	CD
46	(9E,11Z)-13-Oxo-9,11-octadecadienoic acid	26.61	-	295.2268	277.2162 179.1434	$C_{18}H_{30}O_3$	0.95	Fatty acid	(Lu et al., 2014) CD
47	Dihydroxy octadecadienoic acid	26.63	311.2230	-	293.2125 275.2019	$C_{18}H_{32}O_4$	4.29	Fatty acid (DiHODE)	(Lu et al., 2014) CD
48	Dihydroxy octadecadienoic acid	26.76	311.2230	-	293.2125 275.2019	$C_{18}H_{32}O_4$	4.69	Fatty acid (DiHODE)	(Lu et al., 2014) CD
49	Dihydroxy octadecadienoic acid	26.94	311.2231	-	293.2123 275.2020	$C_{18}H_{32}O_4$	4.49	Fatty acid (DiHODE)	(Lu et al., 2014) CD
50	(9E,11Z)-13-Oxo-9,11-octadecadienoic acid (I)	27.40	293.2126	-	275.2016 171.1017	$C_{18}H_{30}O_3$	4.79	Fatty acid	(Lu et al., 2014) CD
51	*tris*-(Dihydrocaffeoyl) spermine	27.41	-	693.3461	293.0565	$C_{37}H_{48}O_9N_4$	-5.43	Alkaloids	(Yossa Nzeuwa et al., 2017, Wang et al., 2018)
52	9-Hydroxy-10,12-octadecadienoic acid	27.86	295.2279	-	171.1018 277.2176	$C_{18}H_{32}O_3$	4.05	Fatty acid (HODE)	(Lu et al., 2014) CD
53	(9E,11Z)-13-Oxo-9,11-octadecadienoic acid (II)	28.24	293.2124	-	275.2015	$C_{18}H_{30}O_3$	4.37	Fatty acid	(Lu et al., 2014) CD
54	Palmitic acid	28.91	255.2330	-	/	$C_{16}H_{31}O_2$	4.07	Fatty acid	(Liu et al., 2021, Bondia-Pons et al., 2014)
55	Linoleamide	29.36	-	280.2636	263.2368	$C_{18}H_{33}ON$	-0.34	Fatty acid Metabolite	(Wang et al., 2023)
56	Palmitic amide	29.84	-	256.2636	88.0761 102.0913 172.1689	$C_{16}H_{33}ON$	0.47	Fatty acid Metabolite	(Wang et al., 2023)
57	Oleamide	30.06	-	282.2789	265.2529 247.2418	$C_{18}H_{36}ON$	0.66	Fatty acid Metabolite	(Wang et al., 2023) CD
58	6-methyl-9-heptadecenoic acid	30.86	281.2487	-	/	$C_{18}H_{34}O_2$	4.09	Fatty acid	(Kumar et al., 2022) CD
59	Protocatechuate	31.01	153.0184	-	109.0283	$C_7H_5O_4^-$	0.89	Phenolic acids	(Liu et al., 2021)
60	Violaxanthin	34.24	-	601.4229	/	$C_{40}H_{56}O_4$	-4.10	Xanthophylls	(Dumont et al., 2020)

CD: Compound Discoverer

4.3.4. LBE Exhibits an Antioxidant Activity on Cancer Cells

The antioxidant activity of goji berry extract was assessed by measuring reactive oxygen species (ROS) levels using 10 µM DCFH-DA. To test the effect of LBE on ROS neutralization, MCF-7 cells were treated for 24 hours at concentrations of 25, 12 and 6 µg/mL (Figure 50). As expected, and in agreement with the results obtained from DPPH,

ABTS and Metal binding assays, LBE significantly inhibited ROS production in the cell medium at all tested concentrations.

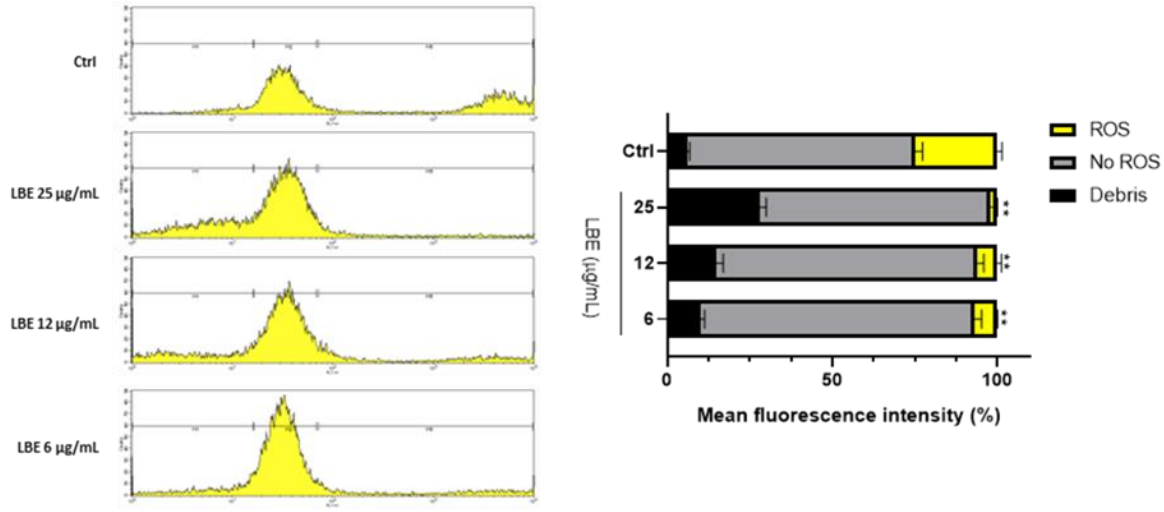


Figure 50. *Measurement of intracellular ROS production evaluated by DCFH-DA via cytofluorimetric analysis on MCF7 cells. To test the effect of LBE (25, 12, 6 µg/mL) to ROS neutralization, MCF-7 cells were incubated for 24 h. Data are expressed as % of M1, M2, M3 (M1 = cell debris; M2 = healthy population; M3 = ROS population). Results are showed as mean ± standard deviation (SD) from three independent experiments. *, ** and *** denote respectively $p < 0.05$, $p < 0.01$ and $p < 0.001$ vs. Ctrl.*

Furthermore, to evaluate the possible antioxidant activity of LBE, and to confirm the previously obtained data, an absolute quantification of the mRNA levels of the NADPH oxidase genes (Nox1-5) was performed by microfluidic droplet digital PCR (ddPCR).

Nox1-5 expression levels were evaluated on both MCF-7 and MCF10A cells. Breast cancer tumor cells show a significant increase in Nox mRNA levels, compared to the levels present in healthy cells (Figure 51), demonstrating that tumor cells exhibit high oxidative stress due, among other things, to the overexpression of Nox genes.

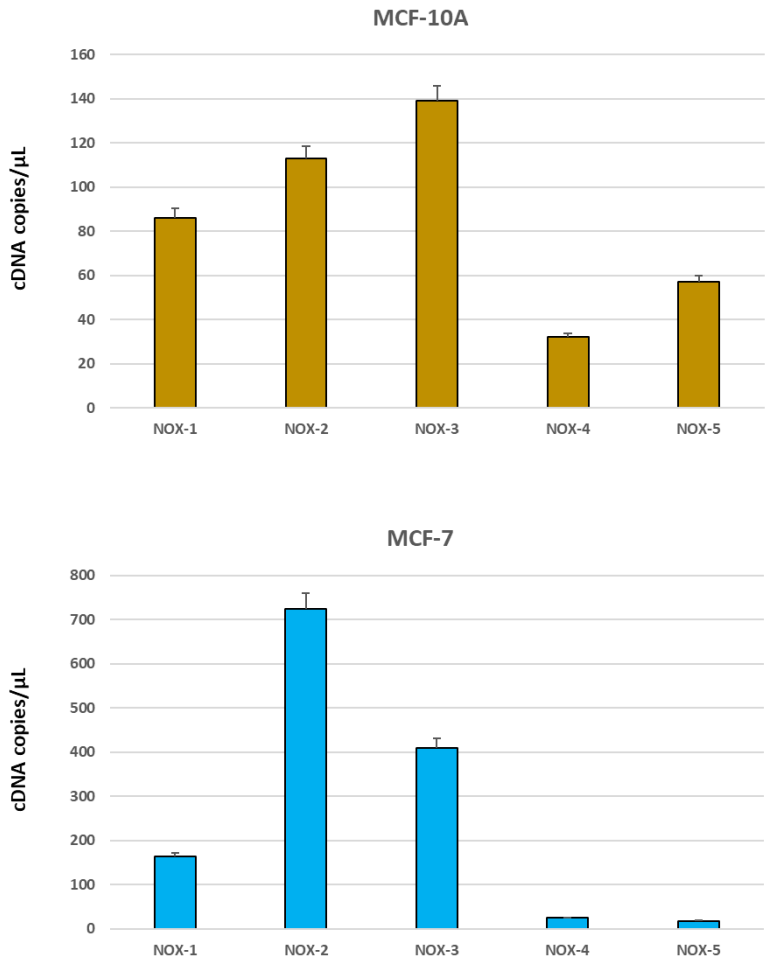


Figure 51. *Absolute quantification of the Nox mRNA expression in MCF10A non-tumor cells (**above**) and MCF-7 cancer cells (**below**). Three independent ddPCR experiments gave the same relative results. Error bars represent Poisson confidence interval at a 95% level.*

In particular, there was a strong increase in the expression of Nox2 and Nox3. There were approximately 110 cDNA copies per µL of the Nox2 transcript in healthy cells while in tumor cells it increased to more than 700 copies. Similarly, the cDNA copies present in MCF10A for Nox3 were 140 copies per µL and approximately 400 copies were found in MCF-7.

Subsequently, the activity of the ethanolic extract of *L. barbarum* on gene expression was evaluated. The cells were treated with LBE extract for 24 h at a chosen concentration of 100 µg/mL. The results show that LBE-treated MCF-7 cells exhibit a significant

reduction in the cDNA levels of all Nox genes. In particular, it was demonstrated that the extract reduced Nox2 and Nox3 cDNA level by approximately 10-fold. The number of copies went from around 700 per µL to less than 70 copies per µL for the absolute quantification of Nox2. Similarly, the expression of Nox3 decreased from approximately 400 copies per µL to less than 40 copies per µL (Figure 52).

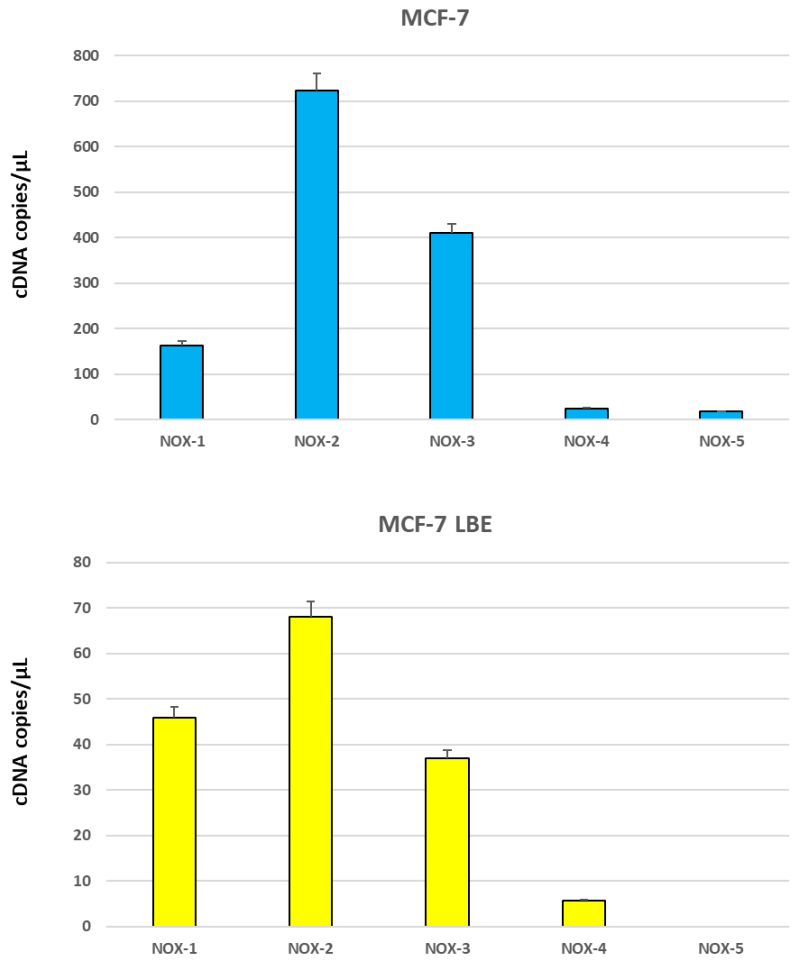


Figure 52. *Absolute quantification of the Nox mRNA expression in MCF-7 cancer cells* ***(above)*** *and MCF-7 cancer cells treated with LBE for 24 h at a concentration of 100 µg/mL* ***(below)****. Three independent ddPCR experiments gave the same relative results. Error bars represent Poisson confidence interval at a 95% level.*

Furthermore, the results show that Nox1, Nox4 and Nox5 also undergo a reduction in the number of cDNA copies per µL, demonstrating that LBE has an important role in

regulating the expression of Nox genes. These data confirm the results obtained with the previous tests and highlight a good antioxidant potential of the *L. barbarum* extract.

4.3.5. Lycium barbarum Extract Induces Cell Death in MCF-7 Cells Saving Healthy Cells

The anticancer activity of goji berry extract has been evaluated on MCF-7 human breast cancer cell line. Cell proliferation was examined with the use of MTT assay. The viability of LBE-treated MCF-7 cells was measured after 24h of treatment, using the extract alone in a concentration range of 3.125–100 µg/mL (Figure 53A). The EC_{50} for MCF7 cells was calculated to be 53.45 ± 3.46 µg/mL, whereas no toxicity was found on MCF10A cells (EC_{50}>100 µg/mL) (Figure 53B). The cytotoxic effect showed by LBE was also evaluated through propidium iodide staining. Hence, flow cytometry analysis was performed to confirm the formation of hypodiploid nuclei, which represent a hallmark of cell death. Our results have shown that goji extract significantly increases the number of hypodiploid nuclei in a concentration-dependent manner in cancer cells (6-25 µg/mL; $p < 0.01$ vs. Ctrl at the highest concentration) (Figures 53C, 53D). To confirm these data, we decided to perform a PI/Hoechst 33342 double staining. Hoechst 33342 is a cell-permeable nuclear counterstain that emits blue fluorescence when combined with the minor groove of dsDNA. On the other hand, PI represents a membrane-impermeable dye that labels death cells that lost their membrane integrity by emitting strong red fluorescence (Carolina et al., 2011). We found that LBE determines significative effect on cell viability in accordance with flow cytometry data (6-25 µg/mL; $p < 0.01$ vs. Ctrl at the highest concentration) (Figures 53E, 53F).

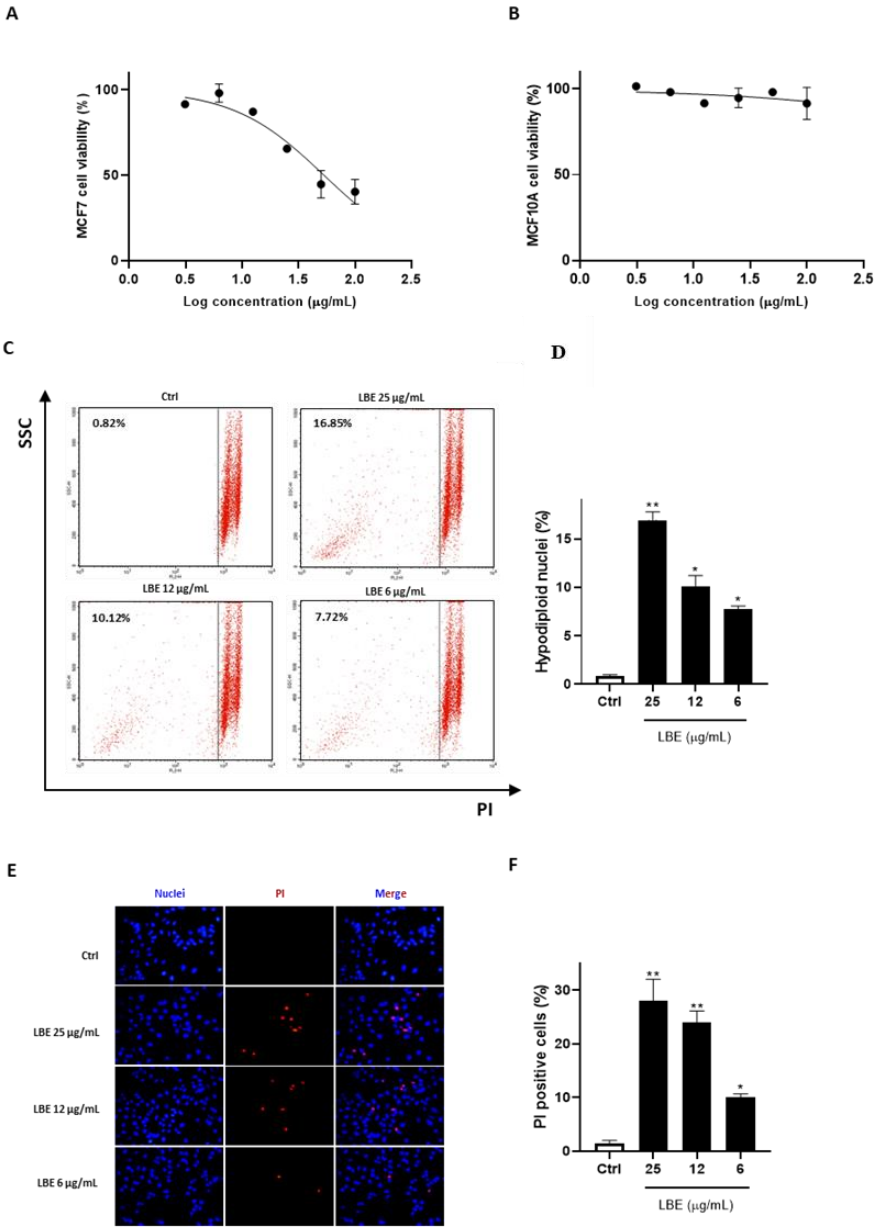


Figure 53. *Cytotoxic effects of LBE towards the breast cancer cell line MCF7. Inhibitory action on the cell proliferation of LBE was measured by MTT assay after 24 h of treatment towards MCF-7 (**A**) and healthy epithelial cell line MCF10A (**B**). Then EC_{50s} were estimated. EC_{50} values are expressed as mean ± SD. (**C**) After 24h of LBE exposure (25, 12, 6 µg/mL), MCF-7 cells were stained by propidium iodide and fluorescence of hypodiploid nuclei (sub G0/G1) was measured by flow cytometry. (**D**) Quantitative analysis of hypodiploid nuclei were reported. (**E**) Hoechst 33342/PI double staining was performed to analyze dead and living cell distributions after the exposure of LBE (25, 12, 6 µg/mL). (**F**) Quantitative analysis of PI positive cells was reported (N≥10). Scale bar: 20 µm. Cells were observed at 20× magnification. Data are showed as the mean ± SD of three different experiments performed in triplicate. * $p < 0.05$ vs. Ctrl; ** $p < 0.01$ vs. Ctrl; *** $p < 0.001$ vs. Ctrl.*

4.3.6. LBE Induces an Activation of Endoplasmic Reticulum Stress

At this point, to elucidate a possible mechanism inducing cell death, the correlation between the cytotoxic effect of LBE and endoplasmic reticulum (ER) stress activation with consequent unfolded protein response (UPR) was examined. Western blots analysis was performed to evaluate the increase of (PKR)-like ER kinase Ser/Thr protein kinase (PERK) expression and inositol-requiring Ser/Thr protein kinase 1α and RNA endonuclease (IRE1α) phosphorylation as well as activating transcription factor 6 (ATF6) cleavage. Short times were considered for the investigation, since the UPR help cells to adapt to such stress within few hours (Ryoo, 2016, Franceschelli et al., 2011, Vestuto et al., 2022). As shown in Figure 54A, LBE (6 µg/mL) caused a significant activation of all three transducers compatible with a general cellular stress condition. As expected, the chaperone Glucose regulatory protein 78 (GRP78), the master regulator of the UPR, results overexpressed compared to control, since it regulates the binding-release of the ER stress transducers, which appear to be activated. Moreover, considering that ER stress response ultimately results in caspase-12-activation (Morishima et al., 2004), we evaluated its activation by the administration of LBE. A notable reduction in its non-cleaved form was observed following LBE administration (Figures 54A, 54B). Considering the strong increase in IRE1α, we further evaluated the activation of its target NLPR3 inflammasome. Furthermore, in the light of endoribonuclease activity of IRE1α, to confirm its activation we performed a q-PCR to evaluate the levels of unconventional mRNA splicing of X-Box Binding Protein 1 (XBP1), a specific transcription factor that undergoes excision of a 26-nucleotide unconventional intron from IRE1α (Vestuto et al., 2022). As depicted in Figure 54D, we found high levels of sXBP1 compared to control ($p < 0.001$). PERK activation was also evaluated by its downstream activation product ATF4 (Activating transcription factor 4) through q-PCR obtaining very high levels of

ATF4 compared to control ($p < 0.001$) (Figure 54D). The remarkable activation of ATF4 transcript, together with c-ATF6 and s-XBP1 activation, is fully compatible with the increased expression of their transcription factor CHOP (C/EBP Homologous Protein), as highlighted in Figure 54A. CHOP protein, also known as GADD153 (growth arrest- and DNA damage-inducible gene 153), is activated as part of the UPR when ER stress is severe or prolonged and triggers cell death (Kamiya et al., 2012, da Silva-Maia et al., 2023). Given that oxidative stress is among the major inducers of ER stress, and further considering the pro-oxidant action that natural extracts, including goji extract, may have toward cancer cells (He et al., 2012, Du et al., 2022), we decided to assess intracellular ROS production following administration of the goji extract. To this end, MCF7 cells were treated with 50, 25, 12 and 6 μg/mL LBE for 1-24 h and subsequently ROS were determined with DCFH-DA administration. As a result, we revealed a significant increase of ROS over a time range of 1-8 h compatible with an insult capable of triggering ER stress (Figure 54C). Moreover, it emerged that LBE has an antioxidant action after 24 hours of treatment on MCF-7 cells confirmed both by the reduced levels of ROS. In addition to this, the expression of the antioxidant factor nuclear factor erythroid 2–related factor 2 (Nrf2), activated in a critical adaptive response to oxidative stress, was evaluated, revealing a time-dependent increase in its expression (Figure 54A). Nrf2 is also activated, *inter alia*, from PERK phosphorylation (Cullinan et al., 2003).

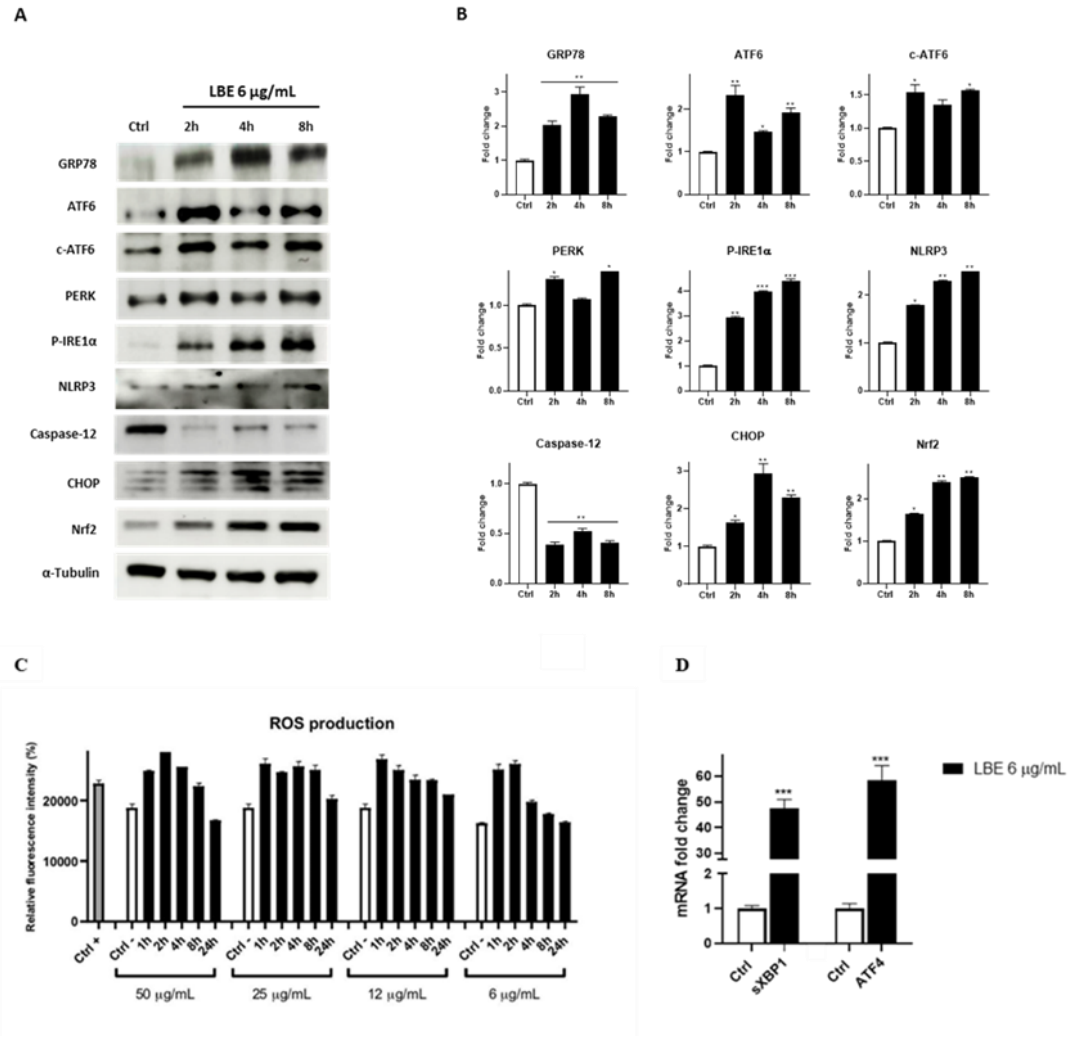


Figure 54. *Biochemical effects of LBE on ER stress pathways. (**A**) Representative western blot images showing increased expression of ER stress markers GRP78, ATF6, PERK, P-IRE1α and some of their downstream target (NLRP3,CHOP, Nrf2). Levels of cleaved proteins involved in the UPR (c-Caspase-12 and c-ATF6) were also evaluated. α-Tubulin was used to check equal loading of protein extracts. The relative fold change vs untreated cells, set as 1, is shown in the graph. (**B**) Densitometric analysis of western blotting. (**C**) Measurement of intracellular ROS detected with DCFH-DA. H_2O_2 (800 µM, 4h) was used as positive control. (**D**) s-XBP1 and ATF4 mRNA ER stress hallmarks were assessed with q-PCR. GAPDH was used as housekeeping control. The $2^{-\Delta\Delta CT}$ method was employed to calculate the relative quantities of mRNA. Results are expressed as fold change relative to untreated cells. Data are showed as the mean ± SD of three different experiments performed in triplicate. * $p < 0.05$ vs. Ctrl; ** $p < 0.01$ vs. Ctrl; *** $p < 0.001$ vs. Ctrl.*

4.4. Discussion

Compared to healthy cells, many tumors produce and maintain high levels of intracellular ROS leading to a state of increased basal oxidative stress that may promote numerous aspects of tumor development and progression. Elevated rates of ROS are due to a redox imbalance caused by their lower levels of antioxidant enzymes and endogenous antioxidants (Senthil et al., 2004, Liou and Storz, 2010). As this state of oxidative stress makes cancer cells vulnerable to drugs that further increase ROS levels, the use of natural pro-oxidant agents is emerging as an exciting strategy to selectively target cancer cells (Shin et al., 2020, Martin-Cordero et al., 2012, D'angelo et al., 2017). Excessive amounts of ROS can start indeed toxic chain reactions, which oxidize and disable the structures necessary for cell survival and proliferation.

In this regard, the action of the polyphenolic extract of *L. barbarum* on breast cancer cells was examined. The extract, rich in polyphenolic substances, has been shown to possess a significant action on raising the intracellular levels of ROS. LBE in the first hours of treatment (1-8 h) demonstrated a strong pro-oxidant action with a high increase in ROS, triggering an imbalance inside the cell which led to disabling the structures necessary for survival and proliferation and induce cell death. Consequently, to the marked pro-oxidant action of LBE, the reduced levels of ROS and the reduction of mRNA levels of NADPH oxidase genes at 24 h were recorded, in accordance with literature (Pérez-Torres et al., 2017, Irazabal and Torres, 2020).

Moreover, several studies have reported that natural compounds can exert a pro-oxidant activity in the presence of metal ions (Yordi et al., 2012). In particular, we hypothesize that the increase of ROS in the first hours of treatment is due to the high presence of transition metal ions such as Fe^{3+} and Cu^{2+}, whose concentration in tumor cells is higher than in normal cells. In the presence of Cu^{2+}, the pro-oxidant activity of

phenolic compounds was supposed to progress to generate hydroxyl radicals (•OH). Meanwhile, extra amounts of Fe^{3+} in a living cell can catalyze the generation of ROS via Fenton reaction, which can damage DNA, lipids, proteins and nucleic acids. Iron ions are generally believed to be essential for several processes related to oxidative stress in natural systems (Eghbaliferiz and Iranshahi, 2016).

In recent years, endoplasmic reticulum (ER) stress has attracted widespread attention as a novel mechanism of cell death induced by natural compounds (Martucciello et al., 2020). To elucidate the mechanism of ROS-induced cell death exerted by LBE, we investigated the possibility of goji extract to trigger cell death via ER stress in MCF-7. Oxidative stress disturbs ER homeostasis leading to the accumulation of misfolded or unfolded proteins, triggering the UPR response (Folch-Puy et al., 2016). The chaperone protein GRP78 is an important regulator of ER stress, apoptosis and cell survival. Under physiological conditions, GRP78 usually combines with three ER transmembrane proteins, respectively, such as PERK, ATF6 and IRE-1, thereby maintaining their inactive state. Once the accumulation of misfolded or unfolded proteins occurs in cells induced by redox imbalance, GRP78 dissociates from these three proteins and initiates ER stress. PERK, ATF6 and IRE-1 can promote the folding of new proteins and remove misfolded and unfolded proteins to restore ER homeostasis through their respective pathways, carrying out an adaptive reaction that favors cell survival, whereas in overactive ER stress, the three proteins drive toward cell death by upregulating cell death markers.

These data have shown for the first time that polyphenolic component of *Lycium barbarum* L. has the ability to inhibit cell proliferation and induce cell death through its pro-oxidant action in breast cancer cell line MCF-7 via ER stress activation, as observed by western blots and q-PCR experiments.

GRP78, the master regulator of UPR, was found to be overexpressed relative to control, as it regulates the binding-release of ER stress transducers, which appear to be activated. Indeed, the extract caused a significant activation of PERK expression, phosphorylation of IRE1α as well as ATF6 cleavage, all three transducers compatible with a general cellular stress condition. Furthermore, caspase-12 and CHOP, downstream proteins of the ER stress response, were also highly activated after LBE treatment demonstrating that the cells exhibited a strong activation of ER stress leading to cell death.

Interestingly, LBE, while showing a cytotoxic effect against tumour cells, is well tolerated by normal cells given the altered microenvironment and the activation of particular molecular pathways present only within cancer cells.

4.5. Conclusion

In conclusion, the main objective of the study was to evaluate the onconutraceutical potential of five plant matrices and in particular *Malpighia emarginata*, *Arbutus unedo*, *Lycium barbarum* L., *Annona cherimola*, *Diospyros kaki*. The potential effects of the polyphenolic extract of *Lycium barbarum* on a breast cancer cell line were studied in more detail, as it was found to be the best extract among the five under investigation in the initial screening. The results obtained showed that goji berry extract is able to reduce the viability of tumour cells without having a significant effect on healthy cells. An interesting aspect that emerged is also a double behaviour on reactive oxygen species, as it has both an antioxidant and a pro-oxidant action. In particular, pro-oxidant action induces activation of ER stress, which could explain a possible mechanism of action by which the extract induces cell death in tumour cells. Taken together, *Lycium barbarum* appears to be a very promising natural medicine in cancer prevention and treatment due to their high content of active compounds. Moreover, considering these findings and that

many chemotherapeutics acts precisely by activating ER stress, the co-administration of these drugs with LBE could represent a new potential therapeutic treatment that aims at enhancing the antitumor action of these drugs but at the same time dampens their side effects in healthy cells thanks to its high antioxidant action.

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