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THE POTENTIAL OF WHEAT AS A FUNCTIONAL FOOD: FROM THE SPROUTS AND MICROGREENS PRODUCTION TO THE NUTRACEUTICAL EFFECTS

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Abstract

Polyphenols are one of the most important phytochemicals derived from plants like fruit, and vegetables, however, wheat are also good sources of polyphenol. More and more evidence in vitro and in vivo experiments showed the health-promoting potential of polyphenols and wheat polyphenols due to their bioavailability correlated to digestion, absorption, and metabolism in humans. Each polyphenol compound has its own unique chemical structure that contribute to specific solubility and lipophilicity, which affects the bioavailability. The biopharmaceutical classification system may be helpful to measure the dietary polyphenol and consequently to understand their nutraceutical potential. Although many studies reported the bioavailability of polyphenols, there is some confused information regarding their lipophilicity and BSC classification. The present study will summarize all the information regarding 10 dietary polyphenols including the main polyphenols of wheat and their association with the BCS classification.

Polyphenol content is affected by the genotype of plants, as well as storage and processing method. Whole wheat flour is a normal product rich in polyphenol components consumed in daily diet, but little attention is paid to its relationship with storage conditions. Therefore, phenolic extracts of whole wheat flour from four cultivars (Verna, Timilia, Sagittario and Claudio) stored for 0, 2, 4, 8 weeks under aerated or vacuum condition were analyzed in present study. Polyphenol content, profile, and antioxidant activities were evaluated. In general, the polyphenol content and antioxidant activity of whole wheat flour decreased after storage for 8 weeks. However, these parameters were not significantly influenced by the storage conditions. Among the wheat varieties analyzed, Verna exhibited the high levels of polyphenol content and antioxidant activity.

Germination is a processing method which can maintain or improve nutritional value of cereal by avoiding milling or baking. Many bioactive compounds are present in wheat sprouts and microgreens that are usually produced in a controlled environment with artificial light. Therefore, the effect of LED and fluorescent light on growth parameters, polyphenol and pigment content as well as antioxidant activities of wheat

sprouts and microgreens from the same four cultivars were evaluated. The effect of light was generally not obvious in wheat sprouts, but much greater and species-specific for wheat microgreens. In general, LED light was beneficial for the increase of polyphenol and pigment contents and antioxidant activities compared to fluorescent light. Verna sprouts showed the highest polyphenol content among the four cultivars. However, Claudio microgreens grown under LED light exhibited the highest polyphenol content and antioxidant properties. All the extracts of wheat microgreens exhibited the potential of bioprotective effects on Caco-2 cell viability.

Although wheat polyphenols were influenced by storage time and light condition when the seeds are germinated for wheat microgreens, our results showed their antioxidant activities and health benefits on human health. It provides more information for developing a new food supplement of wheat polyphenol. However, the data on their safety dose of intake are still very few. In the present study the effects of gallic acid and ferulic acid on viability, proliferation and migration of intestinal, fibroblasts cells and monocytes were respectively determined, as well as on a tridimensional intestinal model. Results of present study showed that ferulic and gallic acids had toxic effects on in vitro cell models with high dose.

Overall, the results of present study validate the effect of the storage time on polyphenol of whole wheat flour, also indicate that LED light should be a good light source for production of wheat microgreens. In addition, it provides more evidence about safe dose avoiding toxic effects on intestinal wall of wheat polyphenol when they will be developed as supplement.

Table of contents

General Introduction 1
1. Dietary Polyphenols and Wheat Polyphenols 1
2. The Effect of Storage on Wheat Polyphenols 3
3. The Effect of Processing and Light Conditions on Wheat Polyphenols 4
4. Safety of Dietary Polyphenol Supplements 6
5. Overview of the Research 7
5.1. Significance 7
5.2. Hypothesis and Objectives 8
Reference 10
Chapter 1 - An Overview on Dietary Polyphenols and Their Biopharmaceutical Classification System (BCS) 13
1. Introduction 14
2. Polyphenol Metabolism 16
2.1. Metabolism Course 16
2.2. PC Metabolites Have Different Biological Activities 17
3. Polyphenol Bioavailability 19
3.1. Hydroxycinnamic Acids 21
3.2. Flavonols 22
3.3. Flavones 22
3.4. Isoflavones 23
3.5. Stilbenes 23
3.6. Tannins 24
3.7. Curcuminoids 25
4. Permeability and Solubility of Polyphenols 26
4.1. LogP 26
4.2. Solubility 30
5. BCS Classification of Polyphenols 33
6. Conclusions 37
References 40
Chapter 2 - Effect of Storage Conditions and Time on the Polyphenol Content of Wheat Flours 52

1. Introduction 53
2. Materials and Methods 55
2.1. Chemicals and Reagents 55
2.2. Wheat Samples 55
2.3. Extraction of Free and Bound Polyphenol 55
2.4. Determination of Total Polyphenol Content 56
2.5. Determination of Total Flavonoids Content 56
2.6. HPLC-MS/MS Analysis 56
2.7. Assay of Antioxidant Activity in Vitro 57
2.8. Statistical Analysis 57
3. Results 57
3.1. Moisture Content 57
3.2. Total Polyphenol Content 58
3.3. Total Flavonoid Content 59
3.4. Analysis of the Phenolic acid Profiles 60
3.5. Antioxidant Activity 61
4. Discussion 63
4.1. Moisture Content 63
4.2. Total Polyphenol and Antioxidant Activity 64
4.3. Phenolic Acids Profile 66
5. Conclusions 67
References 68
Chapter 3 - Effect of Light Condition on Pigments, Phenolic Content, Antioxidant and Bioprotective Activities of Wheat Sprouts and Microgreens 71
1. Introduction 72
2. Materials and Methods 74
2.1. Materials 74
2.2. Growing Conditions 74
2.3. Growth Parameter 75
2.4. Shoot Pigments (Chlorophyll and Carotenoid) Analysis 75
2.5. Total Aqueous Extraction 75
2.6. Cell Viability 77

2.7. Statistical Analysis 77
3. Results 77
3.1. Growth Parameters of Wheat Sprouts and Microgreens 77
3.2. Shoot Pigments of Wheat Sprouts and Microgreens 80
3.3. Polyphenol Contents of Wheat Sprout and Microgreen Extracts 82
3.4. Antioxidant Activity of Wheat Sprout and Microgreen Extracts 83
3.5. The Effect of Wheat Sprouts and Microgreens on Cell Viability of Caco-2 Cells 84
4. Discussion 85
4.1. Growth Parameters of Wheat Spouts and Microgreens 85
4.2. Shoot Pigments of Wheat Sprouts and Microgreens 86
4.3. Total Polyphenol and Antioxidant Activity of Aqueous Extracts 87
4.4. The Effect of Wheat Sprouts and Microgreens on Cell Viability of Caco-2 Cells 88
5. Conclusion 88
Reference 90
Chapter 4 - Are Supplements Safe? Effects of Gallic and Ferulic Acids on In Vitro Cell Models 95
1. Introduction 96
2. Materials and Methods 97
2.1. Chemicals 97
2.2. HPLC-MS/MS Analysis 98
2.3. Cell Model Systems 99
2.4. MTT Viability Assay 99
2.5. Cell Viability 100
2.6. Caspase3/7 Detection 100
2.7. Wound-Healing Assay 100
2.8. Monocyte Migration 100
2.9. Intestinal Equivalents 101
2.10. Immunohistochemical Analysis 101
2.11. Statistical Analysis 102
3. Results 102

3.1. Gallic and Ferulic Acid Content in Commercially Available Dietary Supplements....102
3.2. Effects of Gallic and Ferulic Acids on Mouse Fibroblast Proliferation and Migration....103
3.3. Effects of Gallic and Ferulic Acid on Human Intestinal Cell Viability....105
3.4. Effects of Gallic and Ferulic Acids on Human Monocyte Cell Viability....106
3.5. Linear Discriminant Analysis (LDA)....107
3.6. Effect of Gallic and Ferulic Acids on Intestinal Tridimensional Models....109
4. Discussion....110
4.1. Gallic and Ferulic Acids and Intestinal Tissue Cell Lines....110
4.2. Gallic and Ferulic Acids and Tridimensional Intestinal Equivalent Model 112
4.3. Conclusions....113
References....114
General Conclusion....116

General Introduction

1. Dietary Polyphenols and Wheat Polyphenols

Polyphenols are the most important phytochemicals derived from plants. They share a common structural feature, an aromatic ring with one or more hydroxyl groups [1]. More than 8000 polyphenols have been identified and categorized into four subclasses: flavonoids, phenolic acids, stilbenes and lignans [2]. Polyphenols are secondary plant metabolites and the most common bioactive components obtained from plant in daily diet, which can protect plants from pathogens, ultraviolet radiations and herbivores. They are widely found in fruits, vegetables, tea, coffee and wine, however, cereals and leguminous are also good sources of polyphenols [3].

Regarding the polyphenols present in the wheat grain, phenolic acids and flavonoids are the most abundant ones. Most of these compounds, are present in bound forms attached to the cell wall or in insoluble condition. On the other hand, a small proportion of phenolic acids and flavonoids are existed in free or soluble forms [4]. Polyphenols of wheat are mostly found in the outer three layers of bran (aleurone, testa and pericarp). Ferulic acid is the main phenolic acid present in wheat bran and wholegrain flour, accounting for about 90% of total polyphenols, followed by *p*-hydroxybenzoic acids, *p*-coumaric acid, vanillic acid, sinapic acid, syringic acid, caffeic acid, and protocatechuic acid. The flavonoids are mainly located in the wheat germ [5].

The polyphenol content and profile of wheat depend on many factors, such as variety, climatic conditions and other environment factors, which can, together or singularly, significantly influence them [6]. Many studies have evaluated the polyphenol content and profile of different wheat varieties from Italy, China, Spain, United States, Hungary and other countries. In addition, it was reported that the polyphenol profile of wheat was affected by the cultural environment [6]. Significant differences in the phytochemical fingerprints were observed from ancient and modern wheat varieties of Italy. Six ancient wheat cultivars showed phenolic profiles with higher contents than that found in the modern ones [7]. In contrast, higher phenolic contents were found in modern cultivars compared to the ancient ones among 19 wheat cultivars released or

introduced between 1870 and 2013 in the United States. Moreover, the content of total phenolic acids was significantly different among the various release years [8]. It was also reported that the free form of phenolic compounds from six Italian wheat cultivars was mainly determined by the environment factors (different harvest location and year), while a significant effect of genotype was found in the bound forms, which resulted to be in larger amount in red grain varieties [9]. Significant differences in phenolic acid content of 26 wheat cultivars were observed also in relation of their growing locations (Hungary, France, United Kingdom, and Poland). Moreover, different growth years in Hungary also influenced the free and conjugated phenolic acid contents. Bound phenolic acids were more stable. Some cultivars of wheat were more resistant to environmental factors than others. [10].

The benefits of polyphenols and wheat polyphenols on human health has been widely demonstrated. More and more evidence obtained by *in vitro* and *in vivo* experiments illustrated the health-promoting potential of polyphenols and wheat polyphenols. Polyphenols can protect macro-molecules from reactive oxygen species (ROS) induced damage and exhibit antioxidant activity [1]. A direct effect on endothelial cells has also been demonstrated. The polyphenols from black carrot showed protective effects under inflammatory conditions in endothelial cells [11]. A preventive effect on obesity and diabetes has also been demonstrated. Phenolic compounds from seagrasses of the genus Zostera showed therapeutic action on diabetes and hyperlipidemia [12]. Polyphenols also can improve the cardiac functions. It was, in fact, reported that people with higher intake of polyphenol had a more favorable cardiovascular risk factors profile than the ones with lower intake [13]. In addition, a systematic review provided comprehensive evidence that higher intake of flavonoid-rich foods could be helpful to reduce the risk of colorectal cancer [14]. From this perspective, it is important to further evaluate the nutritional potential of dietary polyphenols and develop dietary polyphenols as new supplements, this may be done through some specific nutritional or biopharmaceutical classification system.

2. The Effect of Storage on Wheat Polyphenols

Storage is one of the most important post-harvest processes, which should be performed properly to make sure the quality and shelf life of food products. During storage, the environmental temperature and relative humidity, as well as the control of storage time, are important parameters to be considered for their commercial value. The content of phenolic acids from food material changed, and a decrease or increase of phenolic acid content was found during storage process. These changes of phenolic acid content was mainly due to storage temperature [15]. Serafini [16] found that the plasma quercetin levels significantly increased after the intake of fresh lettuce and ingestion for 3 h. Conversely, no changes of plasma quercetin levels were observed after the intake of the same amount of lettuce stored under modified-atmosphere packaging for 3 days and the content of quercetin in the lettuce decreased by 64% within this storage time. It was reported that the polyphenol content and the antioxidant activity of hazelnuts decreased after a storage for 12 months, but these parameters were not significantly influenced by the storage conditions (air, vacuum and modified atmosphere) nor from the various temperature (ambient temperature, refrigeration at 5°C, or frozen at –25°C) [17].

A prolonged storage of cereals is possible if the kernels are maintained intact. According to Raki [18], the phenolic content of wheat kernels increased significantly, and the contents of catechin and vitexin-glucoside did not change significantly after 24 months of storage. In addition, the total antioxidant activity was quite stable during storage. In contrast, it was reported an increase of total flavonoid content in the free form but a decrease in the bound part from the black rice grains stored under conventional and vacuum conditions for 12 months. Moreover, a loss of the total phenolics content was observed in all the storage conditions including nitrogen condition [19]. If the grain is ground to flour, cell walls are broken, and structure of the seed is destroyed. The endosperm is exposure to microbes, enzymes and environmental moisture and gases without the protection of outer layers. There are some changes occurred in deterioration processes, such as free radical generation and non-enzymatic

glycosylation, fragmentation of proteins, lipids and sugars, consequently influencing chemical and quality parameters, including organoleptic properties of cereal grains flour. The changes in the flour during storage are probably because of enzymes production or release in the flour. The quality of flour could be destroyed completely if storage for a long period. The moisture contents of grains or flours and the storage temperature and humidity also could influence the acidity and pH of the cereals and flour products. In addition, the changes of phenolics may cause the deterioration of flavor and taste of the food [20].

Phenolic compounds present in grain flour may be involved in some chemical and physical reactions during food storage. Research on the changes of phenolic content and profile of grain flour during storage are very important to understand the mechanisms of their degradation and to develop these ingredients as functional food in the future [21]. A reduction of phenolic content was observed in sorghum flour within the first 60 days of storage, but high retention (more than 89.4%) was showed at the end of storage (180 days) [22]. However, very few studies have focused on the phytochemical and biological activity changes in wheat flour during storage. This aspect necessitates further study.

3. The Effect of Processing and Light Conditions on Wheat Polyphenols

Gemination, also known as sprouting, is a way of processing with improvements of bioactivity and nutritional value of seeds, since it increases the contents of phytochemicals such as phenolic acids, flavonoids, and vitamins [23]. Cereal sprouts and microgreens can maintain nutritional value of grains by avoiding milling or baking. Bioactive compounds including polyphenol of cereal distribute unevenly in kernels, with higher content in aleurone layer, followed by the bran fraction [24]. Germination could prevent from diminishing the bioavailability of essential minerals in cereal because anti-nutritional molecules such as phytates and oxalates are decreased, and other bioactive compounds are produced in the meantime [25]. It was reported by Donkor [26] that the germinated wheat, oats, barley, rye, sorghum, buckwheat and

brown rice exhibited increased content of bioactive compounds including gallic acid, catechin, EGCG, ferulic acid and *p*-coumaric as well as higher antioxidant activities compared to their seeds. Moreover, it was reported by Hung [27] that the content of syringic acid in alcohol extract from two germinated Canadian wheat cultivars was increased, subsequently resulting to increase the total phenolic contents. Eleven phenolic acids were observed in three germinated Polish wheat cultivars, and the contents of *p*-hydroxybenzoic, syringic and *p*-coumaric acids were increased by gemination process [28].

The biosynthesis of polyphenol in plants is affected by many environmental factors, especially for the sprouts and microgreens cultivated normally in a controlled environment, of which the light intensity, spectrum and photoperiod are the main ones during germination processing. For example, the flavonoid content of tobacco leaves was increased with higher proportions of far-red light and decreased with higher UV-A and red/far-red ratio [29]. It was reported that light exposure increased phenolic compound content of soybean microgreens, of which blue light and UV-A significantly increases the content of total phenolic and flavonoid compared to white light control [30].

Artificial light sources, such as fluorescent light and high-pressure sodium (HPS), are usually used in controlled environment for plant production. However, more attention have been paid to LED lights in the past two decades, due to their advantages including the adjustable light spectral composition, flexible light intensity, high energy efficiency, long lifetime, low heat emitting, and environmental-friendly property. LED lights with a combination of red (600 – 700 nm) and blue (400 – 500 nm) light are commonly used for plant grown under controlled environments. It is well known that chlorophylls and other pigments like carotenoid possess a large absorption in the red and blue light regions [31]. It was reported by Liu [32] that the total phenolic and flavonoid contents of the pea sprouts grown under blue and red LED light, were higher than that grown under the dark environment. Moreover, the contents of all the individual phenolic compounds of the pea sprouts grown under blue LED light were

significantly higher than that grown under the dark control. Benincasa [33] found that a lack of significant effect of light on wheat sprouts were observed. However, the effect of light was significantly different and species-specific for wheatgrass: the contents of free and total polyphenols, tannins, flavonoid and phenolic acids increased for einkorn wheatgrass grown under blue light and emmer wheatgrass grown red light.

4. Safety of Dietary Polyphenol Supplements

Various kinds of polyphenols are found in plant or natural products. Usually, people can have a daily intake of dietary foods with high content of polyphenols, such as fruits, vegetables, tea, wine and coffee [34]. Much evidence showed that polyphenols intake can benefit human health. For example, polyphenols intake has prevention effects on cardiovascular disease, obesity, diabetes and aging, and also can improve the ability of human brain cognition [35]. However, polyphenol content is highly different in various food. Within a specific food, polyphenol content also can differ significantly due to the cultivation environment, post-harvest storage condition and processing method. Given the clear health benefit and difference of various polyphenols, there is an increased interest in developing new dietary supplements containing polyphenols for public use. In addition, specific polyphenols may be purified and/or concentrated, singly or a combination with several polyphenols, to improve the health condition of the consumer. More and more efforts have been made to improve the bioavailability of specific polyphenols, and to increase polyphenol concentrations in plasma and tissue levels, which the efficiency is higher than the commonly consumption with dietary foods [36]. Therefore, the consumption of such supplements can increase daily intake, absorption and ultimately plasma concentrations of polyphenols and potentially confer their health benefits.

However, the upper limit of doses for daily intake and polyphenols concentrations in plasma level avoiding adverse effects is unknown for many polyphenols. Many evidence confirmed the hypothesis that high-dose polyphenols intake could cause side effects on *in vitro* models, animals and human by pro-oxidative action. Resveratrol, a

typical polyphenol present in red wine, is available developed as a dietary supplement. It was reported that there was no evidence shown any toxicity effects on the rats treated with a resveratrol supplement (Longevinex) and commercial resveratrol, which suggested the beneficial effects and safety of resveratrol [37]. However, it was reported that resveratrol at concentrations of 0.1 – 1000 μmol/L could increase the growth of breast cancer cells [38]. It was well-known that green tea is safe and healthy beverage with abundant antioxidants, however, a high dosage of green tea would result an injury on bone matrix due to becoming a prooxidants [39]. In addition, it was reported that high doses of (2)-epigallocatechin-3-gallate (EGCG), which was main compound of green tea, has minor side effects on humans like thirst and diarrhea [40]. Moreover, the cell viability of hepatocytes was significantly decreased by treatment of high-dose EGCG (200 μmol/L), and the ROS production was increased [41]. Ferulic acid, which is mainly found in the leaves and seeds of many cereals such as whole wheat, brown rice, oats, coffee and peanut with antioxidant potential, exhibited an anti-cancer potential to tongue, skin and colon when fed to rats at 500 mg/kg diet, but was carcinogenic to liver [42].

Although the function of polyphenols could be promoted by including in a new pharmaceutical formulation that can work directly with specific targets, obviously avoiding adverse effects, regulated market of commercial polyphenol supplements and nonpharmaceutical formulations should be considered for their safe application [43].

5. Overview of the Research

5.1. Significance

As polyphenols are bioactive compounds with many benefits on human health, it is important to study their digestion, absorption and metabolism within human body. More specifically, a nutritional biopharmaceutical classification system should be applied for the purpose of evaluation their nutraceutical and therapeutic potential. In addition, the polyphenols of plants are not totally stable, easily degraded during storage after harvest and their biological activity may be altered. However, few studies have focused on wheat seeds or flour. For this perspective, it is important to proceed a study on the

changes of polyphenols and biological activity of wheat flour, especially the effect of oxygen and time for storage. Moreover, polyphenols are also affected by processing method, for example, an increase of polyphenol content of seeds is observed after germination. Regarding the production of cereal sprouts and microgreens, light is one of the most important factors for plant growth, which can influence the biosynthesis of phytochemicals, such as polyphenols and chlorophylls. However, the research on the effect of light on wheat sprouts and microgreens are not fully understood. It's important to conduct a further study of the effect of different light conditions and gemination time on the wheat. Since polyphenols can promote human health, a daily intake should be guaranteed. The consumption of polyphenol supplements can increase daily intake, absorption and ultimately plasma concentrations of polyphenols thus potentially improving human health. However, the safety dose for daily intake is an important factor that should be considered when some specific polyphenols are developed as supplements.

5.2. Hypothesis and Objectives

The overall hypothesis of the thesis was to evaluate if the polyphenol content of four wheat varieties could be affected by different storage and processing methods. In particular, the four wheat varieties, were chosen modern and ancient from one side and soft and durum from another side. The overall objective of this work was to analyze if, the storage and processing method could influence the wheat polyphenol content according to the specific proprieties of the wheat variety selected. A series of experiments were designed to investigate the effects of storage time and conditions, light used for production of microgreens on the polyphenol contents and antioxidant activities of wheat flour and microgreens. In addition, a further study to confirm the safety use of wheat polyphenols to support the future development as supplements was performed. In particular, the specific objective of this work of thesis were the followings:

1) To summarize the information of metabolism, bioavailability, permeability, solubility and BCS classification of 10 dietary polyphenols including the main polyphenols present in wheat from relevant progressive studies, to discuss the

correlation among these factors, and to evaluate the nutritional potential of these dietary polyphenols.

2) To investigate the effects of storage time (0, 2, 4, 8 weeks) and conditions (aerated and under vacuum) on the total phenolic and flavonoid contents, composition of phenolic acids, and antioxidant activities of wholegrain flours from four different wheat cultivars (Verna, Timilia, Sagittario and Claudio).
3) To evaluate the effect of LED and fluorescent light on the growth parameters including shoot length, fresh weight and dry matter, pigments (carotenoids, chlorophyll a and b) contents, total phenolic and flavonoid contents, as well as antioxidant and bioprotective activities of sprouts and microgreens from four wheat cultivars (Verna, Timilia, Sagittario and Claudio) grown for 6 days.
4) To assess the effects of ferulic (main polyphenol of wheat) and gallic acids on the viability, proliferation and migration of three different cell lines (Caco2, L929 and U937), which somehow represent the principal cellular component of the human intestinal tissue, as well as on a tridimensional intestinal model containing mucosal layer and a submucosa with fibroblasts and monocytes.

Note: Each chapter in this thesis is an independent manuscript which has either been published (Chapter 1 in International Journal of Molecular Sciences, Chapter 2 in Processes Journal, Chapter 4 in Nutrients Journal) or submitted to journal (Chapter 3).

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Chapter 1 - An Overview on Dietary Polyphenols and Their Biopharmaceutical Classification System (BCS)

Abstract

Polyphenols are natural organic compounds produced by plants, acting as antioxidants by reacting with ROS. These compounds are widely consumed in daily diet and many studies report several benefits to human health thanks to their bioavailability in humans. However, the digestion process of phenolic compounds is still not completely clear. Moreover, bioavailability is dependent on the metabolic phase of these compounds. The LogP value can be managed as a simplified measure of the lipophilicity of a substance ingested within the human body, which affects resultant absorption. The biopharmaceutical classification system (BCS), a method used to classify drugs intended for gastrointestinal absorption, correlates the solubility and permeability of the drug with both the rate and extent of oral absorption. BCS may be helpful to measure the bioactive constituents of foods, such as polyphenols, in order to understand their nutraceutical potential. There are many literature studies that focus on permeability, absorption, and bioavailability of polyphenols and their resultant metabolic byproducts, but there is still confusion about their respective LogP values and BCS classification. This review will provide an overview of the information regarding 10 dietary polyphenols (ferulic acid, chlorogenic acid, rutin, quercetin, apigenin, cirsimaritin, daidzein, resveratrol, ellagic acid, and curcumin) and their association with the BCS classification.

Keywords: polyphenols; metabolism; bioavailability; LogP; biopharmaceutical classification system

1. Introduction

Phenolic compounds (PCs) are secondary plant metabolites, characterized by an aromatic ring and several attached hydroxyl groups. These compounds offer protection to the plant from pathogens, free oxygen radicals, UV rays, and parasites [1].

Polyphenols represent a large and varied group of at least 10,000 known different compounds that could be unified by the presence of one or more aromatic rings with one or more hydroxyl groups in their chemical structure [2,3]. For some plant products, for example, some exotic fruits or cereals, the composition of polyphenols is still poorly known [4]. Regarding dietary PC, the current known compounds are about 8000 variants and they are naturally found in common fruits, vegetables, and beverages and, according to the number of phenolic rings they contain, they could be classified into main four subclasses: [5] flavonoids, including flavonols, flavones, isoflavones, flavanones, anthocyanidins, and flavanols; phenolic acids subclass, which is divided between those compounds derived from hydroxybenzoic acids, such as gallic acid, and those derived from hydroxycinnamic acid, like caffeic, ferulic, and coumaric acid; and stilbenes and lignans (Figure 1) [6,7]. In addition to these, there are other subclasses that are not included among the currently known, i.e., alkylphenols, curcuminoids, furanocumarins, hydroxybenzaldehydes, hydroxybenzoketones, tyrosols, and so on [8].

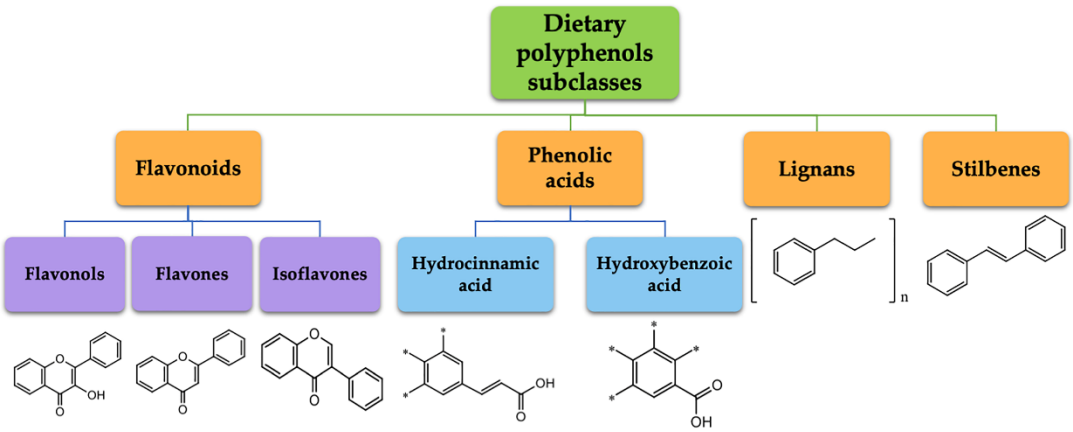


Figure 1. Dietary polyphenols known subclasses scheme.

PCs are derived from a common biosynthetic pathway, involving precursors from the shikimate and/or the acetate-malonate pathways [9]. In addition, their role in the prevention and improvement of human health has been widely demonstrated. There is growing evidence to support antioxidant, anti-inflammatory, anti-tumoral, and anti-cardiovascular disease roles attributable to polyphenols [10–18]. There are numerous different types of PCs, all characterized by different chemical structures that have distinct properties [19]. It was reported that the specificity of the health benefits, conferred by a single PC, is based on specific chemical classes [20].

As PCs are bioactive compounds, it is important to study their distribution within the human body. More specifically, in order to express their therapeutic effect, PCs should undergo pharmacological metabolism reactions, with consequent conversion into more soluble metabolites, and subsequent excretion [21,22]. From this perspective, it is important to study the health potential of PCs, and this may be done through the biopharmaceutical classification system (BCS). This classification evaluates the capacity of drugs (and thus also bioactive compounds) to pass through lipidic biological membranes as well as to interact with aqueous solutions during their metabolism within the human body. Hence, BCS allows to characterize the health capacities of a PC.

The aim of this work is to analyze the nutraceutical potential of 10 dietary PCs, in terms of intestinal absorption, permeability, solubility, and BCS classification of these compounds, and to compare them with the literature currently available, in order to establish an association between all these factors. Furthermore, it was chosen to focus this work only on 10 dietary PCs because they belong to most of the subfamilies of known polyphenols, and thus can be representative for an overall evaluation of the beneficial prospective of dietary PC present in nature. Besides, in the current literature, there is enough and exhaustive information on permeability, solubility, and especially BCS classification only on these PCs, univocally directing the discussion of this review to them.

2. Polyphenol Metabolism

2.1. Metabolism Course

In mammals, PCs are subject to both phases I and II of drug pharmacokinetic metabolism, respectively, as represented in Figure 2. PCs are ingested mainly in a conjugated form, as O-glycosides (step 1, Figure 2). Metabolism of glycosylated PC is initiated in the oral cavity [22], after contact with the glycosidase enzymes of oral microflora, as demonstrated by Kamonpatana et al. [23] for anthocyanidins. However, most PCs continue intact along the digestive tract [22]. On arrival in the stomach and inside the small intestine mucosa, the glycosides are converted by a hydroxylation reaction into their corresponding aglycones (phase I drug metabolism) (step 2, Figure 2). This reaction is assisted by β-glucosidase enzymes expressed by the intestinal microbiota. In this way, aglycones may pass from the gut lumen to the cytosol of the enterocytes, predominantly by passive diffusion (step 3, Figure 2), or by protein carriers, such as P-glycoprotein (P-gp) and co-transporters for sodium-glucose transporter (SGLT1) [24–26]. Some hydroxycinnamic acids, such as ellagitannins, are resistant to enzymatic digestion in the small intestine and, therefore, pass directly to the colon, where they are metabolized by microbiota into aglycones [27]. Once the aglycones have been absorbed into the enterocytes or colon cells, they move through the portal vein (step 4, Figure 2) to the liver, where they are further conjugated (phase II drug metabolism) to become O-glucuronides and O-sulphates (step 5, Figure 2). A variable portion of the phenolic conjugates is then excreted into the bile and re-enters the small intestine to undergo the metabolic cycle once again [28–30]. Finally, the resultant phenolic conjugates (O-glucoronides/O-sulphates) are transported to the bloodstream by plasma proteins until they are excreted in urine [31–33].

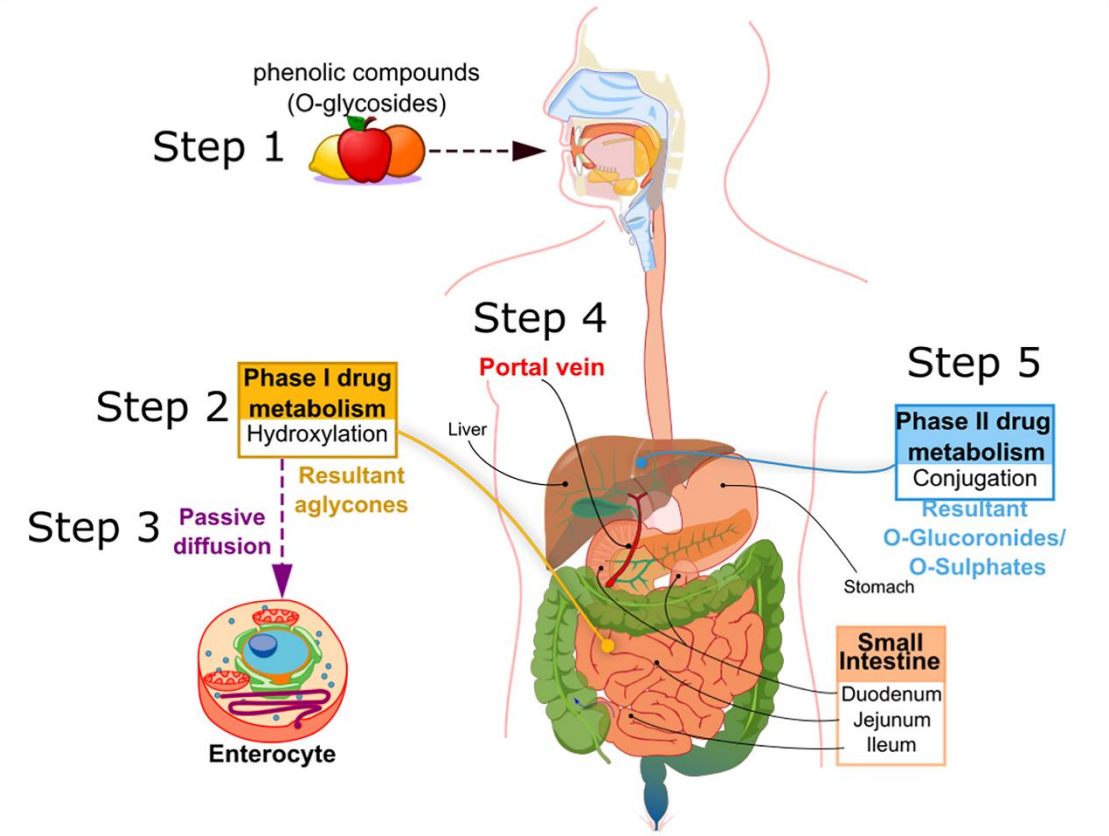


Figure 2. Principle metabolic steps of dietary phenolic compound (PC) in humans.

2.2. PC Metabolites Have Different Biological Activities

Biotransformation, during the metabolic processes in humans, is determined by the structural characteristics of the specific phenolic compound [9]. This is because the chemical structure of the compound is specific in promoting the action of only selected intestinal enzymes and gut microbiota species. It has been shown that gut microbiota are involved in the release of phenolic aglycones and hepatic O-glucuronides [28,34]. According to the different structural subfamilies, PCs undergo intestinal bio-transformations by specific microbiota families [31,35–40]. Furthermore, it was observed that each single phenolic compound metabolized generates numerous metabolite byproducts, usually two or three, but also many more, i.e., glycosylated quercetin produces up to 20 metabolites. According to Del Rio [41], all these modifications during absorption have a profound influence on the biological activity of the resultant phenolic metabolites, as these may play an active role within different

pathways in the human body. An example is the activation of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) [42]. It was shown that protocatechuic acid, a metabolite from anthocyanins, is a known Nrf2 activator [43,44]. Likewise, caffeic acid metabolites have Nrf2 activating properties [45–47]. In addition, another protective function pathway was found to be influenced by phenolic metabolites; it was shown that methylated scutellare presents an inhibitory effect on H_2O_2-induced cytotoxicity in PC12 cells, thus indicating protective activity [48]. In order to be able to perform all the metabolic reactions, it is necessary that PC, as well as all xenobiotics, administered orally, enter the intestinal epithelium to reach the blood and lymphatic circulation [49]. The transcellular mechanisms required to permit the entry of these compounds into the intestinal mucosa are as follows: passive diffusion, carrier-mediated active facilitated transport, and paracellular transit in tight junctions [50,51]. Nevertheless, generally, the majority of drugs enter the cells by passive diffusion [52]. PCs, characterized by low molecular weights, and that are sufficiently hydrophobic and non-charged, are permitted to be transported by passive diffusion [24]. This involves the production of biliary salts and the formation of micelles, which permeate through the translocation of the apical membrane of the enterocytes [53]. Some PCs, such as hydroxytyrosol, tyrosol, *p*-coumaric acid, apigenin, and luteolin, are selectively combined in micelles and absorbed differently [54]. In addition, in an in vitro study using Caco-2 cells to test the permeability of six dietary polyphenols (caffeic acid, chrysine, gallic acid, quercetin, resveratrol, and rutin), it was shown that several chemical-physical features are related to the passive diffusion transport capability of molecules through cells. These factors include the lipophilicity (expressed as partition coefficient logarithm, LogP), molecular weight, ionization state the number of rotatable bonds (RB), and number of hydrogen-bonding acceptor/donor (HBA/HBD), respectively [55]. Moreover, it was observed that the scarcely lipophilic ferulic acid (FA) passes through transcellular transport, by tight junction [56]. Regarding structurally complex PCs, such as gallotannins and ellagitannins, hydrolysis processes permit conversion into smaller molecules, thereby facilitating assimilation in simpler

forms by enterocytes. However, this conversion reaction cannot occur in the small intestine, so they pass directly into the large intestine where they are fermented by the microbiota and then can be absorbed by passive diffusion at the level of the colon [9,57,58]. All these transport mechanisms influence the bioavailability of these compounds in humans [49].

3. Polyphenol Bioavailability

Bioavailability is a term used in pharmacokinetic language to indicate the fraction of the drug that reaches the systemic circulation without any chemical modification. Recently, it has also been used for food nutrients. According to the current accepted definition, bioavailability is the proportion of the nutrient that is digested, absorbed, and metabolized through normal pathways [54].

Each class of PC has its own unique chemical structure that results in specific solubility and lipophilicity, which in turn affects the bioavailability. These parameters influence both the rate and degree of absorption of phenolic metabolites in the human body during metabolism, before being incorporated into plasma circulation [59]. In the studies of Marrugat [60], Fito [61], and Tian [62], the bioavailability of various PC was analyzed by detecting plasma and urine concentrations after the ingestion of pure compounds or food with a known phenolic content. It was found that phenolic concentrations in either plasma or urine were not directly related to the respective concentrations in target tissues, but were instead dependent on their metabolic form. This is explained by the fact that PC, although ingested in a glycosylated form, undergo metabolic processes, such as hydrolysis/hydroxylation by the intestinal enzymes and subsequent conjugation with either glucuronic or sulfonic acid in the liver. This leads to a change in their chemical structure, thereby modifying the respective lipophilicity and solubility characteristics, permitting entry into the blood circulation. Therefore, PCs are found in the blood circulation in conjugated forms. To confirm this, quercetin and daidzein aglycones were not found in either plasma or urine after they are ingested, but in their conjugated form (after phase II metabolism) [63]. Resveratrol represents an

exception, as it undergoes glycosylation in order to protect the compound from oxidative degradation. Hence, the glycosylate resveratrol is chemically more stable and soluble, and consequently more easily absorbed in the human gastrointestinal tract [64]. Finally, it was shown that large size conjugated metabolites are eliminated in bile, while small conjugates, such as monosulphates, are preferably excreted in urine [59].

In this perspective, BCS classification may allow prediction of health effects for PC contained in foods and thus are administered mostly orally. In 2000, the Food and Drug Administration (FDA) purposed the BCS system as an approach to avoid in vivo tests when drugs are also characterized by rapid dissolution [65]. More in particular, it was highlighted that high permeability of a drug does not limit the absorption of a compound during its transit in the intestinal system. In addition, high solubility of a drug will not limit its dissolution and consequently neither its absorption, thus the gastric emptying process is the only limiting step for the absorption of a highly soluble and highly permeable compound [66]. However, some studies have shown that the FDA's BCS guidance, despite supporting studies on bioavailability through in vitro test such as on Caco-2 [66], may not always be enough to correctly predict the extent of drug absorption in humans [67,68].

To study the bioavailability of an active compound, the most reliable measure is the area under the plasma drug concentration curve versus time (AUC). AUC is directly proportional to the total amount of unchanged drug that reaches systemic circulation. Plasma drug concentration increases with the extent of absorption, and the maximum plasma concentration is reached when the drug elimination rate equals the absorption rate. The time peak, which is reached when maximum plasma drug concentration occurs, is the most widely used general index of absorption rate; the slower the absorption, the later the peak time. To determine significant AUC values, a cutoff of 80% was defined [69]. For dietary supplements, herbs, and other nutrients, such as PC, in which the administration is nearly always oral, bioavailability can be identified as the quantity or fraction of the ingested dose that is absorbed [70]. Hence, in this review,

the maximum plasma concentration and time peak of the PC examined will be taken into consideration for the evaluation of their bioavailability.

3.1. Hydroxycinnamic Acids

In the early study of Lempereur [71], it was seen that the source of hydroxycinnamic acids in foods is relatively varied. For instance, FA is the most abundant phenolic acid found in cereal grains and represents up to 90% of the total polyphenol content. In the study of Zhao [72], it was shown that FA has a very high bioavailability in rats [73,74]. It was observed that FA, after undergoing absorption by intestinal epithelial cells and conjugation reactions [75], was present in both plasma and urine mainly in its conjugated form [76]. In several clinical studies carried out on rats, FA administrated at 8 μmol kg^{-1} [72], and between 6 and 15 mg kg^{-1} [77–79], displays high plasma concentration between 8174.55 ng L^{-1} and 0.444 mg L^{-1}. Higher results were detected with a higher dosage (20 mg kg^{-1}) [80], with a peak plasma concentration of around 12 mg L^{-1}, whereas it seems that even higher doses (0.5 and 1.5 g kg^{-1}) lead to a maximum plasma concentration of around 12 ng mL^{-1}, showing a limiting rate of absorption. Furthermore, FA time peak was observed at less than 1 h, which implied that it is rapidly absorbed in rat plasma after oral administration [81].

Caffeic acid is the most representative hydroxycinnamic acid present in nature, and can be found in food (mostly fruits) as well as in its ester form, as chlorogenic acid [19]. Clinical studies observed that chlorogenic acid is rapidly absorbed and metabolized by the intestine, thus the digestion metabolites may be detected in plasma between the first 5 min and 1 h after ingestion in rats [82,83]. The current literature showed that chlorogenic acid absorption is dependent on administered doses, i.e., a low administered dose (1–100 mg kg^{-1}) [83–85] leads to a concentration between 0.55 and 91 ng mL^{-1} of caffeic acid in plasma concentration; likewise, higher doses (400 mg kg^{-1} from Lonicerae Japonicae Flos extracts) [82], for which peak plasma concentration was registered at around 1500 mg mL^{-1}, show an absorption rate dependent on the ingested dose. Regarding urinary excretion, the concentration of chlorogenic acid found after 24 h from ingestion was around 30–34%, indicating that this compound is rapidly eliminated as well as it is rapidly absorbed [83].

3.2. Flavonols

The most studied flavonols are rutin and quercetin, and they are mostly found in buckwheat, asparagus, and citrus fruits, but also in peaches, apples, and green tea [86,87]. In pharmacokinetics studies carried out on rats and human volunteers [88–92], low bioavailability of rutin was shown, due to its hydrophilic nature, thus suggesting that it cannot diffuse easily through cell plasma membranes. To be absorbable, rutin needs to undergo to hydroxylation into quercetin. In fact, after oral administration of rutin (328 μmol kg^{-1}), only quercetin sulfates and glucuronides were detected in serum, with a concentration of 2 and 5 nmol mL^{-1} [89]. This evidence was in line with previously observations, where, after oral rutin administration (500 mg), the absorption rate was 40 – 200 ng mL^{-1} of quercetin [88] and showing the metabolism changes that occur on this compound, which are necessary to absorb it within the human body.

Quercetin bioavailability was found to be very poor, as it is rapidly metabolized in the human body; therefore, in the conjugated form (quercetin metabolite), its beneficial capacities are limited compared with the aglycon form [91]. Furthermore, the total quercetin conjugates measured in plasma concentrations after oral ingestion are very low. In the study of Dong [90], rats showed rapid absorption of quercetin (8.51mg kg^{-1}) from *Matricaria chamomilla* L. extract, with a final plasma concentration around 0.29 μg mL^{-1} detected after 0.79 h (47 min) from ingestion, confirming that it is rapidly absorbed after oral administration. In Graefe study [91], human volunteers who ingested 100 mg of quercetin glycosides showed a maximum plasma concentration at 2.12 μg mL^{-1} and urine concentration at 4.5%. This evidence confirmed the rapid absorption of quercetin aglycone, which is supposed to occur in the upper part of the intestine, thus involving active absorption mechanisms. Kaşıkcı [93] demonstrated that absolute bioavailability (2.01 μM) of quercetin was attained after ingesting the compound suspended in aqueous solution.

3.3. Flavones

Regarding flavones, celery, red peppers, chamomile, mint, parsley, rosemary, oregano, traditional Chinese herbs, and ginkgo biloba are the major sources of this subclass [94]. It was shown that apigenin administered at both low doses (13.82 mg

kg^{-1}) [90] and high doses (60 mg kg^{-1} and 100 mg kg^{-1}) [95,96], is similarly absorbed, with max plasma concentration registered between 0.14 and 1.33 μg mL^{-1}. Moreover, in the study carried out on six men, the apigenin conjugates detected in urine after 24 h from the administration lead to an excretion rate around 0.22%, suggesting that most of the apigenin ingested is rapidly metabolized or is excreted unabsorbed, thus showing high permeability of this PC within the human body [97].

Following the flavones subclass, cirsimaritin, mostly found in rosemary and oregano, was also investigated. In a pharmacokinetic study on rats [98], 8 mg kg^{-1} of cirsimarin (glycosyde form) was administered from crude extract of Microtea debilis and, 5 h after in-gestion, the low permeability of this PC was demonstrated: the determination of plasma concentrations of cirsimaritin aglycone was 0.138 μM and the urine concentrations after 5 h from oral administration were only 5.05 μM (3–5%). These results showed that cirsimarin is not absorbed from the gastrointestinal tract, but in the stomach, and then it must be converted to cirsimaritin to produce systemic healthy effects within the human body.

3.4. Isoflavones

Isoflavones are present almost exclusively in leguminous plants, particularly daidzein, which is found in large quantities in soybeans and soymilk [99]. Based on the several studies carried out on rats or human volunteers, daidzein showed low bioavailability. In fact, in experiments with both low concentration (from 0.4 to 1 mg kg^{-1}) [100,101] and high concentration of oral administered daidzein (from 30 to 50 mg kg^{-1} and 418 μmol L^{-1}) [102–104], the serum peak registered (173.1 ng mL^{-1} and from 0.38 to 2.5 μmol L^{-1}) appeared within 2 or 8 h from ingestion [102], highlighting its rapid absorption from the gastrointestinal tract, and indicating the low bioavailability of this compound.

3.5. Stilbenes

Despite the low quantities of stilbenes in the human diet, resveratrol is both the most representative polyphenol and the widely studied, as it is considered the main one responsible for health benefits [105–107]. More in particular, it was indicated by numerous recent studies that resveratrol presents several benefits to human health, such

as antibacterial, antioxidant, anti-inflammatory, and anticancer activities [108–111]. Resveratrol has been detected in numerous plants, particularly in red grapes, and thus is highly concentrated in red wine and grape juice [112]. Regarding bioavailability of this compound, after oral administration, resveratrol is absorbed by passive diffusion or by membrane transporters within the intestine, where it undergoes metabolic reactions, and then the resulting metabolite is released in the bloodstream, where it can be detected [113]. It was demonstrated from several clinical studies that, after oral dose of resveratrol between 25 and 150 mg [114–119], the max plasma concentration registered ranges between 491 ng mL^{-1} and 471 μg L^{-1}. Moreover, lower doses of resveratrol were studied (0.5 and 1 mg) [120] and a low plasma concentration of the glucuronidated form at 130.19 ng mL^{-1} was registered, whereas for higher doses (from 500 mg to 5 g) [119–122], the plasma concentration detected was between 0.5 and 4 μg mL−1. These several studies demonstrated that the ab-sorption rate is dependent on the orally administered dose of resveratrol. Urinary excretion of this PC and its metabolites was rapid, with 77% of all urinary agent-derived species excreted within 4 h after the lowest dose [121]. As previously explained, when resveratrol is taken orally, it is metabolized to its glycosylated form, which increases its stability and solubility, allowing this compound to be more easily absorbed [64]. Hence, it was concluded that the systemic bioavailability of resveratrol is high, showing high permeability within the human body, thus accumulation of potentially active resveratrol metabolites may produce healthy effects within the human body [115].

3.6. Tannins

Ellagic acid is a natural phenolic antioxidant found in many fruits and vegetables, such as walnuts, pecans, cranberries, raspberries, strawberries, grapes, peaches, and pomegranates. Clinical studies carried out on this compound showed its low bioavailability. More in particular, both low oral administration (20 – 25 mg) [123,124] and high oral administration (85.3 mg kg^{-1} and > 500 mg) [125,126] showed a plasma concentration in human patients between 30 and 200 ng mL^{-1}. An exception can be made for the 40 mg dose, which can be considered halfway between a low and high administered dose. The serum peak concentration was registered around 200 ng mL^{-1},

which was similar to the serum peak concentration obtained from a high dose of ellagic acid (> 500 mg). These results showed that the absorption system of ellagic acid becomes saturated above a certain dosage of this PC and, therefore, the maximum detectable plasmatic concentration seems to reach its plateau, which appears to be around 200 ng mL^{-1} in the case of ellagic acid. Moreover, this peak concentration in the serum was reached after 1 h from the administration, owing to the fact that 50% of total ellagic acid was shown to bind to blood proteins after intestinal absorption (within the first 30 min after oral administration) [127].

3.7. Curcuminoids

Among Curcuminoids, curcumin covers relevance in Southeast Asia, as it is abundantly found in turmeric, which is a spice widely used in Southeastern Asian countries' culinary traditions [128]. It is relevant to note the highly lipophilic nature of curcumin (high LogP), attributable to the methine-rich segments that connect the polar regions [129]; this is also reflected in its capacity to interact with biomembranes [130,131]. However, its therapeutic potential is still debated owing to its poor bioavailability in humans. Curcumin has been shown to have low permeability and to be poorly absorbed from the small intestine, whereas conjugative metabolism in the liver is extensive [129]. The low availability is also caused by the binding of curcumin to enterocyte proteins, altering its chemical structure [132]. It was seen that curcumin is ineffectively transported through the intestinal mucosa into circulation [133]; this molecule can undergo biotransformation within the intestinal mucosa or directly in the bloodstream [134,135].

Pharmacokinetic studies demonstrated that a high dosage of this compound, between 8 and 12 g daily [128,136–139], displayed a low plasma concentration, ranging between 50 ng mL^{-1} [139,140] and 2 μg mL^{-1} [128], and similar with low dosages (from 100 mg to 4 g daily) [136,137,140–146], with peak plasma concentrations registered around 0.51 nM [136,143], 15.8 nmol L^{-1} [140], and 12.2 and 96 ng mL^{-1} [144–146]. In both studies of Mahale [143] and Dhillon [139], the maximum plasma concentration was detected 2 h after ingestion of curcumin and urinary levels collected after 24 h were 210 and 510 nmol L^{-1} of curcumin glucuronides [140], showing that its metabolites

have a short time period within the human body, meaning curcumin is not expected to be able to exert its beneficial health effects.

4. Permeability and Solubility of Polyphenols

BCS classifications of drugs bioavailability are based on their assigned value of permeability and solubility; if, by one side, the definition of solubility is clear and well established, the permeability concept needs some insights. Apart from active mechanism of trans-membrane selective transport, passive diffusion is the fundamental mechanism for the entry of a drug into human cells, and thus the resulting functional performance. From the perspective of studying the permeability of an orally administered bioactive compound, its diffusion capacity within the human organism tissues is the key property. For this reason, it is fundamental to evaluate the capacity of these compounds to pass through lipidic biological membranes as well as to interact with aqueous solutions (such as gastric acids and bile acids in the gastrointestinal tract). Measurable chemical-physical parameters of a given molecule that can be easily associated with diffusional processes in those environments are aqueous solubility and lipophilicity. Generally, the most lipophilic molecules show scarce water solubility [52]; however, it is necessary that a potentially bioactive molecule shows a specific balance between water solubility and lipophilicity to be biodisposable. A useful measure to define this equilibrium is the partition coefficient, or its logarithmic expression, LogP.

4.1. LogP

Even if lipophilicity of a specific compound is a function of several independent parameters (i.e., molecular mass, non-polar surfaces, total number of H-bond donor and acceptor, ionizable groups, and so on), it is commonly known that the LogP value can be an easy measure to express and compare this property [147]. The LogP value is the base 10 logarithm of the ratio called the partition coefficient, the ratio between the concentrations at the equilibrium of a substance dissolved in a biphasic system 1-octanol/water. The higher the LogP value, the more lipophilic the molecule; conversely, LogP values smaller than 1 are related to hydrophilic behavior. It is relevant to underline that to state that the LogP value is directly related to lipophilicity is a strong

simplification, and some more accurate descriptors have been developed to better describe molecular features. For example, LogD (distribution coefficient logarithm) is basically the same concept as LogP, but it takes into account the ionization capability of some molecules over the whole pH scale. LogD is formally a pH-dependant LogP measure that can describe more precisely the lipophilic feature of a drug dissolved in the bloodstream (pH 7.4) rather than in gastric acids (pH 1–2). Nevertheless, the LogP value, partly for its simplicity, is still considered a precious indicator to take into account in pharmaceutical and environmental chemistry. As an example, one of the empirical rules that describes a potential bioactive drug, formulated in 1997 by Lipinski [148] and some successive updates, identifies a LogP value included in the −0.4 to 5 range as suitable for a drug to be useful pharmacokinetically useful. Moreover, the LogP value is abundantly used as a molecular descriptor in quantitative structure–activity relationship (QSAR) calculations for many purposes.

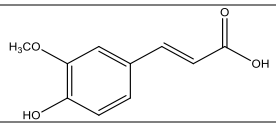
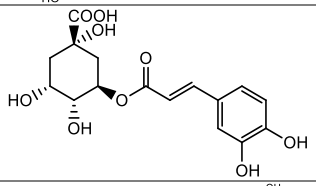
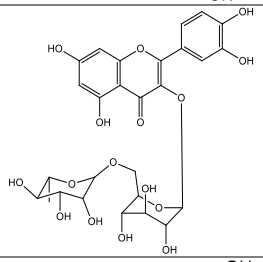
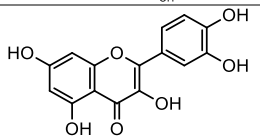
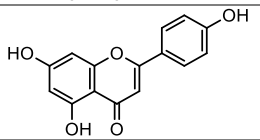
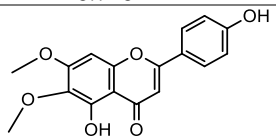
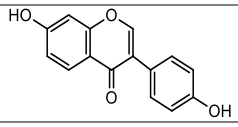
It has to be mentioned that LogP values have not been experimentally determined for any known compounds and, often, calculated values are reported. Because many algorithms are disposable, it is possible to observe a discrepancy between different sources; it is recommended to check several reliable databases to verify the likelihood of the retrieved LogP magnitude order, as well as to compare data obtained from the same mathematical approach. As an example, experimental LogP values of different PCs, reported by the Drugbank database and different references, are shown in Table 1 [55,149–153]. The experimental LogP values of three dietary polyphenols (quercetin, rutin, and resveratrol) were reported by Rastogi et al. [55], analyzed by reverse phase high-performance liquid chromatography (RP-HPLC). Liu [150] reported dissimilar LogP values of the same polyphenols from blueberries using the ChemBioDraw Ultra 11.0. In addition, differing LogP values of apigenin and cirsimartin from rosemary were similarly reported using the ChemDraw software1 Ultra version 8.0 [149] compared with the Drugbank database and other studies. From Table 2, it is also noticeable that the experimental LogP values reported for the considered PCs are in the 0.15 to 3.62 range, with a mean value of 1.99. The most hydrophilic appears to be chlorogenic acid

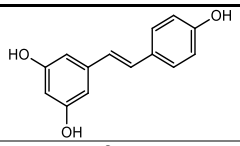
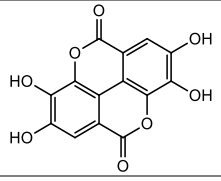
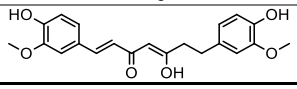
(small molecules with an ionizable acid function), together with rutin, a glycoside coupled with the disaccharide rutinose. The aglycon of rutin, the flavonolquercetin, shows a dramatic rise in lipophilicity (1.7 points in log scale) as a consequence of the loss of two sugar units. All the reported flavonoids (quercetin, apigenin, cirsimaritin, and daidzein) show similar LogP values, while the most lipophilic compound in the list iscurcumin, characterized by aromatic rings conjugated with insaturations, higher non-polar surface, and few polar groups in their chemical structure compared with other considered PCs. Regarding BCS permeability classes, the recommendation set by the United States Food and Drug Administration (USFDA) guide was to consider an absorption value of ≥ 90% as highly permeable [154]. This was subsequently revised, and the value was lowered to 85%, according to the World Health Organization (WHO) guide [155]. Metoprolol is commonly used as a reference [151], because its gastrointestinal membrane permeability lies in this boundary range. For this reason, Metoprolol LogP (1.8 according to Drugbank.com, accessed in April 2021) was used as a boundary marker in order to classify compounds as highly permeable (LogP value ≥ 1.8) or scarcely permeable (LogP value < 1.8). Relaying on this convention, ferulic acid, chlorogenic acid, rutin, and ellagic acid are considered scarcely permeable, while quercetin, apigenin, cirsimaritin, daidzein, resveratrol, and curcumin are highly permeable.

Regarding the LogP value, it may be considered as a helpful instrument to investigate drugs' bioavailability, but effective absorption and metabolism mechanism are much more complex, and specific enzyme and sites interaction also play a relevant role. As an example, it was shown that emodin and chrysophanol exhibited different in vitro activities compared with apigenin and resveratrol [156]. This was attributable to differences in chemical structures, which have different affinities for drug-metabolizing enzymes and transporters, and P-gp, which are found in the intestinal epithelial cells. In particular, it was found that resveratrol absorption involves cis and trans isomers, which are characterized by both a different chemical stability and a differing interaction with intestinal transporters. Based on these properties, resveratrol is highly permeable.

Furthermore, it was seen that the free hydroxyl groups of apigenin and resveratrol cause very rapid glucuronidation and sulfation reactions (phase II drug metabolism, Figure 2, step 5) in the liver or in the intestine, which leads to the production of the same quantity of glucuronidated and sulphated metabolites, respectively. Conversely, chrysophanol and emodine are present in the plasma mainly in the form of glucuronidated metabolites, compared with sulfonated metabolites [93]. A feasible explanation is that the methyl groups present in chrysophanol and emodine impede the production of the sulphated metabolite, and thus increase the possibility of interaction between the hydroxyl groups and the glucuronide enzyme.

Table 1. Experimental LogP values reported by Drugbank.com database compared with miscellaneous references.

Compound	Structure	Drugbank LogP	Other sources LogP
Ferulic acid		1.58	1.42 [150]
			0.96 [151]
Chlorogenic acid		0.17	0.37 [151]
			−0.3 [153]
Rutin		0.15	0.47 ± 0.01 [55]
			−2.28 [150]
			−0.9 [151]
Quercetin		1.81	1.59 ± 0.06 [55]
			0.35 [150]
			1.82 ± 0.32 [152]
			1.48 [153]
Apigenin		3.07	1.51 [150]
			1.9 [149]
			2.92 ± 0.06 [152]
Cirsimaritin		N/A	2.04 [149]
Daidzein		3.3	2.13 [150]
			2.51 ± 0.06 [152]
Resveratrol		2.57	2.99 ± 0.13 [55]
			3.06 [150]

			3.1 [153]
Ellagic acid		1.59	1.366 http://www.chemspider.com (accessed on 28th April 2021)
Curcumin		3.62	3.29 [153]

4.2. Solubility

Solubility (S) is the concentration of a substance at equilibrium in a saturated solution in the presence of excess undissolved solid. In other words, solubility of a solute is the maximum quantity that can be dissolved in a certain quantity of solvent or solution at a specified temperature [157]. Considering that water solubility allows a drug an easy way of subministration, the diffusion capability in aqueous environments of the body, and the attainment of a desired drug concentration in systemic circulation, it clearly promotes the therapeutic activity of a specific compound [158]. Solubility of a given compound in a solvent is influenced by so many parameters that an exhaustive dissertation of the argument is outside the of this review; nevertheless, it is worthwhile to mention that S is dependent on pH, temperature, pressure, polarity of solvent and solute, ionization, enthalpy (solid phases and dissolution), H-bond formation, complex formation, and so on. Solubility can be expressed by different units, involving mass or volume ratios between solute and solvent, but the most used and officially established are concentrations units such as molarity [mol L^{-1}] (moles of solute per liter of solvent) or mass/volume ratio [g L^{-1}] (grams of solute per liter of solvent, and multiples). The magnitude of water solubility ranges widely among all the known compounds, from totally soluble (without limit) such as ethanol, miscible with water in every proportion, to nearly insoluble, such as silver chloride. A number of other descriptive terms have been developed to qualify the extent of solubility in particular fields. For example, U.S. Pharmacopoeia qualifies substances in terms of mass part of solvents required to dissolve one mass part of solute: very soluble (< 1), freely soluble (between 1 and 10), soluble (between 10 and 30), sparingly soluble (between 30 and 100), slightly soluble

(between 100 and 1000), very slightly soluble (between 1000 and 10,000), and insoluble (>10,000) [158]. BCS classification concerning solubility in-volves dividing substances into two classes: highly soluble or lowly soluble. Class boundaries are defined by the capability of the highest single immediate-release dose to dissolve into a specific volume of water (derived from typical bioequivalence study protocols, which prescribe administration of a drug product to fasting volunteers with a glass of water). A drug is considered highly soluble when the highest dose strength is soluble in 250 mL of water over a pH range from 1 to 6.8 (7.5 according to the FDA) [65] at 37 °C. In general, the regulatory authorities consider an active pharmaceutical ingredient (API) highly soluble if its highest dose (D) on solubility ratio (D/S) is less than 250 mL. The former European Medicines Agency guideline (EMA, formerly EMEA, 2001) and the present U.S. FDA (2000) guidance define dose as the highest dosage strength marketed as an oral immediate release (IR) dosage form; that is, the tablet or capsule with the highest content of API. However, the revised EMA (2010) guideline defines dose as the highest single oral IR dose recommended for administration in the Summary of Product Characteristics (also known as the Prescribers' Information). The WHO has a more flexible definition. If the API appears on the WHO Model List of Essential Medicines (EML), the highest dose recommended in that list is to be applied for D/S ratio calculation. For APIs not on the EML, the highest dosage strength available on the market as an oral solid dosage form is used [159]. Clearly, the concept of "the highest dosage strength marketed as an oral IR dosage form" cannot be applied to dietary active principles. With the aim of referring to an analogous value, a sort of "single dietary abundant dose" concept is now proposed and used to determine PC solubility classification. The richest dietary source and the average content value is identified in the literature for any active principle; in the case of PC, www.Phenol-explorer.eu (accessed on 28th April 2021) is particularly useful. For any food categories (i.e., fruits, spices, vegetables, and so on), a weight value marked as "dietary abundant dose" is defined by analyzing consumption statistical data: it is the average daily consumption of an aliment from the highest consuming region of the world. As an

example, in the case of fruits, Dominican Republic was found to be the highest consuming region, with an average of 125 kg per year per capita, equivalent to 342.5 g a day [160]. Similarly, representative food portionz have been determined for chocolate (31.5 g), spices (4.05 g), and processed soy (150 g). These values, multiplied for the average food content for any substance, are used as highest "single oral IR dose" or "single dietary abundant dose" (SDAD). Using the same criterion, SDAD values have been determined for any considered PC. In Table 2, water solubility, richest source, and D/S ratio (calculated as described) values are reported. According to the D/S ratio obtained from the reported assumptions, ferulic acid, chlorogenic acid, rutin, quercetin, apigenin, and cirsimaritin are classified as highly soluble, while daidzein, resveratrol, ellagic acid, and curcumin are considered scarcely soluble, showing a value greater than 250 mL.

Table 2. Water solubility, sources, calculated single dietary abundant dose (SDAD), and calculated dose on solubility ratio (D/S) values of principle phenolic compounds.

Compound	Experimental Water Solubility	Richest Source	SDAD	D/S
Ferulic acid	0.78 mg mL^{-1} [182]	Dark chocolate (240 mg kg^{-1})	7.56 mg	9.69 mL
Chlorogenic acid	40 mg mL^{-1} [153]	Plums (758.8 mg kg^{-1})	259.9 mg	6.49 mL
Rutin	0.13 mg mL^{-1} [161]	Capers (3323 mg kg^{-1})	13.45 mg	103.46 mL
Quercetin	0.0003 mg mL^{-1} [162]	Dark chocolate (250 mg kg^{-1})	7.87 mg	26.23 mL
Apigenin	0.00216 mg mL^{-1} [162]	Origanum majorana (44 mg kg^{-1})	178.2 µg	82.5 mL
Cirsimaritin	2.57×10^{-4} mg mL^{-1} [149]	Origanum vulgare (684.3 mg kg^{-1})	2.77 mg	10.78 mL
Daidzein	0.008215 mg mL^{-1} [162]	Tempeh (processed soy) 136 mg kg^{-1}	20.4 mg	2483.2 mL
Resveratrol	0.03 mg mL^{-1} [162]	Cranberry, red (30 mg kg^{-1})	10.27 mg	342.3 mL
Ellagic acid	0.0093 mg mL^{-1} [162] 0.0097 mg mL^{-1} [163]	Chestnuts (7354 mg kg^{-1})	2.518 g	270752 mL
Curcumin	11×10^{-6} mg mL^{-1} [162]	Curry powder (2853 mg kg^{-1})	11.55 mg	10,500,000 mL

SDAD is an artifact proposed in order to make up for the absence of established dose values for dietary active principles. Moreover, Fong et al. [151] proposed a similar approach to extend BCS classification to Chinese herbal traditional medicine. Moreover, SDAD calculations are revealed to be more consistent than a different strategy considered to solve the following question: referring to acceptable daily intake (ADI) values, calculated on the "no observed adverse effect level"(NOAEL), which is

the higher concentration subministered that reveals no toxic effect on organisms, it can possibly give a representative measure of a “high dosage”. Nevertheless, PCs demonstrated a low toxicity and, consequently, high ADI values that automatically would lead to cataloging any PC as insoluble.

5. BCS Classification of Polyphenols

The BCS was first introduced for the development of new drugs as an aid to guide drug discovery [57]. Based on this system, both permeability and solubility are the two main factors influencing the level of drug transport through the cells of the intestinal mucosa, as well as the relationship between the concentration and the degree of absorption of the respective drug [149,154,164–167]. According to Papich [168], the solubility of a compound within biological compartments, as well as its permeability through biological membranes, limit both the degree and the extent of oral absorption of a drug.

The classification of specific chemical compounds using the BCS system permits an objective evaluation of their bioavailability for the human organism. According to the BCS, drugs can be classified into four categories: Class I (high solubility, high permeability), Class II (low solubility, high permeability), Class III (high solubility, low permeability), and Class IV (low solubility, low permeability). Regarding the four different BCS categories, drugs belonging to Class I have a high rate of both absorption and solubility. Hence, Class I drugs rapidly undergo first-pass metabolism, resulting in reduced concentrations that are then not bioavailable to the organism. Class II drugs are readily absorbed, but have a low solubility rate, which limits the in vivo absorption of the substance, except when administered at high doses. However, the absorption of these drugs is slower than Class I drugs and, therefore, they act over a longer period of time. For Class III drugs, the low permeability results in a limited absorption into the body. As these drugs are highly soluble, they are rapidly dissolved and, therefore, their activity is not dependent on the dose. Finally, Class IV drugs, having both low solubility and permeability, are problematic for effective oral administration, owing to their

limited bioavailability [155,165,169–171]. Although the BCS was originally developed for orally administered drugs, it may also prove to be useful for bioactive substances contained in food, such as PC. In the following Table 3, the information concerning the BCS of PCs examined in this review is gathered, divided between those collected from the current literature and those based on the solubility and permeability criteria evaluated during this work.

Table 3. Biopharmaceutical classification system (BCS) classification of phenolic compounds (PCs) based on the solu-bility and permeability criteria evaluated in this review and based on the current literature.

Family	Compounds	BCS Classification	
		Reviewed	**Literature**
Hydroxycinnamic acid	Ferulic acid	III	III [151]
	Chlorogenic acid	III	III [151]
Flavonols	Rutin	III	III [151]
	Quercetin	I	I [151] or II [172] or IV [173]
Flavones	Apigenin	I	II[174]
	Cirsimaritin	I	III[149]
Isoflavones	Daidzein	II	IV178]
Stilbenes	Resveratrol	II	II [175]
Tannins	Ellagic acid	IV	IV [176]
Curcuminoids	Curcumin	II	IV [177]

Examining the current literature on this topic, PCs are generally characterized by a low solubility and high permeability, i.e., they belong mainly to BCS Classes II and IV, representing a limitation to their potential health benefits [162]. In addition, no PCs assigned to BCS Class I have been found. As shown in Table 3, resveratrol is considered as belonging to BSC Class II, as it is characterized by low water solubility and high lipophilicity [175]. In fact, resveratrol showed a rapid phase II drug metabolism within enterocytes and hepatocytes, owing to the fact that the -OH groups are engaged in labile acetal linkages [178]. Because of this, the bioavailability of resveratrol is limited and, consequently, its health benefits [110].

Similar to resveratrol, apigenin was also indicated as belonging to BCS Class II [174], whereas rutin, FA, and chlorogenic acid were shown to belong to BCS Class III [151]. The metabolism of these compounds was shown to be influenced by cellular absorption, requiring active transport mechanisms to be effectively absorbed by enterocytes [179]. In vitro studies revealed that FA is rapidly absorbed, but the excretion rate is low, and

only 64% of the glucuronide metabolite is metabolized. Likewise, long retention times for FA in vivo were recorded, thus its activity time is prolonged in the human body [180]. Furthermore, according to Perez-Sanchez et al. [149], cirsimaritin was also shown to belong to BCS Class III.

Regarding curcumin, other authors placed it in Class IV of BCS classification because of its poor solubility and intestinal permeability. In fact, it is shown that low plasma and tissue levels associated with curcumin administration are due to its poor aqueous solubility, poor absorption, rapid metabolism, and rapid systemic elimination [177]. Daidzein is a PC commonly assigned to BCS Class IV [181]. Because of its low solubility and poor bioavailability, daidzein may have limited pharmaceutical value. It is further reported that daidzein shows a weak acidity and low permeability [152]. Finally, as reported by Nyamba et al. [176], ellagic acid seems to belong to BCS Class IV.

Controversies have been found in the literature regarding quercetin. In particular, it was shown to belong to either BCS Class I, II, or IV by Fong [151], Madaan [172], and Waldmann [173], respectively. As reported by different studies, this could be attributed to the type of dietary food from which the compound was extracted and analyzed, thereby influencing the bioavailability and biopharmaceutical classification.

Following the solubility criteria purposed by this review, and according to the definitions of high and low permeability and solubility explained in the previous paragraphs, it was possible to elaborate a new BCS classification for the compounds examined in this work. It emerged that polyphenols such as quercetin, apigenin, and cirismaritin belong to BCS Class I, as they are characterized by both a high LogP value (permeability) and high solubility. Quercetin and apigenin fully display the features of this class, showing both fast absorption and excretion, while cirsimaritin behaves more like a BCS Class III drug. It is relevant to mention that, in the case of quercetin, the BCS classification is questionable, given the fact that the available LogP values (reported in Table 1) lie around the edge of the reference parameter (1.8). Regarding BCS Class II, daidzein, resveratrol, and curcumin were identified as belonging to this

class, as they were shown to have a high permeability, but poor solubility. Daidzein and resveratrol demonstrate coherent behavior, showing fast absorption and slow excretion, resulting in a low peak concentration, but long retention time within the organism; contrarily, curcumin shows scarce bioavailability owing to low permeability. Despite the LogP value, curcumin permeability is affected by interactions with gastrointestinal mucous membrane proteins, acting as a barrier. Displaying low permeability and high solubility, FA, chlorogenic acid, and rutin are assigned to BCS Class III. FA shows fast metabolism and excretion rate and non-dose-dependent peak plasmatic concentration, as expected; class III substances, on the other hand, demonstrate fast absorption, which may be explained by diffusion through enterocytes tight junctions. Chlorogenic acid also confirms the BCS Class III characteristic of fast excretion paths. Rutin has been assigned to BCS Class III, but its elevated hydrophilicity represents a limit for absorption, resulting in low bioavailability Finally, only ellagic acid, characterized by low permeability and solubility, is placed in BCS Class IV, well in agreement with in vivo data, stating low bioavailability and the fast reaching of a plateau in systemic concentration.

In conclusion, comparing the data obtained from the criteria proposed by this review with those gathered from the literature, there is good correspondence for FA, chlorogenic acid, rutin, resveratrol, and ellagic acid. Differently, the compounds highlighted in orange in Table3 (apigenin, cirsimaritin, daidzein, and curcumin) are found to belong to a different class than those reported by the literature. This may be explained by the fact that the LogP values of these PCs are obtained through different databases, each of which uses different algorithms, leading to obtaining values of LogP (and thus permeability) that differ from each other. Moreover, referring to the SDAD parameter purposed by this review may lead to doses values sensibly different from those considered in other works.

6. Conclusions

Polyphenols have been widely studied for their several health benefits, and they also are used and examined from nutraceutical point of view, such as supplements. From this perspective, their distribution within the human body and their classification in pharmacological terms lead the interest of this review on the BCS classification of this compounds. Moreover, existing controversies in the literature regarding their classification further increase the interest of this review in evaluating and clarifying the currently available data.

Approaching the BCS classification on dietary bioactive principles, such as PC, immediately raises the problem that there is no univocal definition of maximum strength dose for these compounds. This leads to confusion because each author adopts an arbitrary criterion, or reports data from other studies without considering their context and even omitting necessary information. This is accompanied by variability in LogP values, as they are reported by the various databases as being calculated by different algorithms rather than experimentally verified. All of this results in an indetermination of the boundaries that should define the four classes provided by the BCS system. It would be recommended to identify univocal criteria, which may allow to define the administrable dose of dietary bioactive compounds, consequently allowing to establish, through unambiguous methods, the BCS class for these compounds as well, which are mainly assumed through diet. Nevertheless, the classification proposed by this review largely overlaps with the data found in previous literature, whereas controversies and mismatches largely arise from the definition of the criteria adopted to establish the administrable dosage. It should be kept in mind that the BCS classification was created to study the behavior of active principles purposely subministred exclusively for therapeutic use, while dietary PCs, although manifesting bioactivity on human health, are taken without a purely pharmaceutical purpose.

However, for the 10 PCs examined, the BCS classification seems to be consistent with the bioavailability data found in the current literature. Checking PCs attributed to BCS Class I, quercetin and apigenin fully display the features of this class, showing

both fast absorption and excretion, while cirsimaritin behaves more like a BCS Class III drug (LogP value may possibly be unreliable). Concerning BCS Class II attribution, daidzein and resveratrol demonstrate coherent behavior, showing fast absorption and slow excretion, resulting in low peak concentration, but long retention time within the organism; contrarily, curcumin shows scarce bioavailability owing to low permeability. Despite the LogP value, curcumin permeability is affected by interactions with gastrointestinal mucous membrane proteins, acting as a barrier. Ferulic acid shows fast metabolism and excretion rate and non-dose-dependent peak plasmatic concentration, as expected; class III substances, on the other hand, demonstrate fast absorption, which may be explained by diffusion through enterocytes tight junctions. Chlorogenic acid also displays the BCS Class III characteristic of fast excretion paths. Rutin has been assigned to BCS Class III, but its elevated hydrophilicity represents a limit for absorption, resulting in low bioavailability. Only ellagic acid has been assigned to BCS Class IV, well in agreement with in vivo data, stating low bioavailability and the fast reaching of a plateau in systemic concentration.

Abbreviations

BCS Biopharmaceutical classification system

PC Phenolic compound

P-gp P-glycoprotein

SGLT1 Sodium-glucose transporter

Nrf-2 Nuclear factor (erythroid-derived 2)-like 2

LogP Partition coefficient logarithm P

RB Rotatable bonds

HBA/HBD Hydrogen-bonding acceptor/donor

FA Ferulic acid

FDA Food and Drug Administration

AUC Area under the curve

LogD Distribution coefficient logarithm D

QSAR Quantitative structure-activity relationship

RP-HPLC Reverse phase high-performance liquid chromatography

USFDA United States Food and Drug Administration

WHO World Health Organization

N/ANot available

S Solubility

API Active pharmaceutical ingredient

D Highest dose

D/S Highest dose and solubility ratio

EMA European Medicines Agency

IR Immediate release

EML List of Essential Medicines

SDAD Single dietary abundant dose

ADI Acceptable daily intake

NOAEL No observed adverse effect level

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Chapter 2 - Effect of Storage Conditions and Time on the Polyphenol Content of Wheat Flours

Abstract

Whole wheat flour possesses many nutritional properties because of its abundant bioactive components which are affected by cultivar, but little attention is paid to its relationship with storage conditions. In this study, phenolic extracts of whole wheat flour from four cultivars stored under different conditions (aerated and under vacuum) and different times (0, 2, 4, 8 weeks) were obtained. The total polyphenol (TPC) and flavonoid (TFC) contents, composition of phenolic acids, and antioxidant activities (AA) of phenolic extracts were evaluated. The results showed that Verna exhibited the highest levels of TPC, TFC, and AA for both storage conditions among the four cultivars. Moisture content, TFC, and AA fluctuated during storage. After 8 weeks, the TPC, TFC, and AA decreased with respect to Week 0 in all the cultivars. The TPC losses ranged between 16.39% and 20.88% and TFC losses from 14.08% to 31.18%. The AA losses were approximately 30% from the DPPH assay, but no significant losses were shown in the FRAP assay. However, these parameters were not distinctive between the two storage conditions. The wheat phenolic acid profiles were influenced more by storage time than storage conditions in all cultivars. Overall, the results validate the effect of the storage time on wheat polyphenol.

Keywords: whole wheat flour; phenolic extracts; storage conditions; storage time; antioxidant activity

1. Introduction

Wheat is one of the most important food materials in people's daily diet worldwide, providing calories from carbohydrates and proteins. There is increasing interest in the health benefits associated with the consumption of whole grain food, related to the presence of fiber and bioactive phytochemicals, such as phenolic acids, tocopherols, carotenoids, and so on. Most of these bioactive compounds are found in the outer layer of the wheat kernel (aleurone, bran, and germ), thereby forming the ingredients of whole wheat flour and, in so doing, providing more antioxidant benefits than refined flour [1]. Increasingly more evidence has shown that whole grain flour may help to reduce the risk of cardiovascular disease, chronic diseases, and cancer [2,3].

Phenolic compounds are cited as the most important contributors to antioxidant activity in wheat. These components, which contain hydroxyl groups connected to aromatic rings, can efficiently react and stabilize free radicals [4]. Phenolic compounds are secondary plant metabolites containing one or more aromatic rings attached to one or more hydroxyl groups [5]. Phenolic acids in wheat are mainly present in three forms: soluble free acids, soluble conjugated forms with sugars or other low molecular weight compounds, and insoluble bound forms which are connected to the fiber fraction [6]. Hydroxycinnamic acids are the dominant phenolic compound in whole wheat, among which ferulic acid is the most abundant type, accounting for 70 − 90%, followed by caffeic acid, *p*-coumaric acid, and sinapic acid. There are also benzoic acid derivatives which include protocatechuic acid, *p*-hydroxybenzoic acid, salicylic acid, vanillic acid, and syringic acid [6].

The content of these bioactive components is affected by genotype, cultivar, and the part of the grain sampled. There are many studies on the effects of genotype and environment on phenolic acid content and profiles in wheat and wheat products [7]. Many different factors influence the content of these compounds, such as stress conditions, both biotic and abiotic [8]. A few stresses can significantly increase their content [9]. In addition, a dwarf or semi-dwarf gene was introduced to modern wheat which led to high yields and improvement of disease and pest resistance [5] rather than

of nutritional properties. Nowadays more attention has been given to health benefits from introducing bioactive components in the daily diet, leading to a renewed interest in the selection of varieties, including ancient cultivars, regarding their nutritional potentials. According to the report of Loreto et al. [5], significant differences in the content of phenolic acids were observed between ancient and modern durum wheat. Gotti et al. [7] found that ancient varieties of common wheat showed higher amount of the main represented phenolic acids than modern ones.

Phenolic compounds are not totally stable and are easily degraded during storage after harvest. Subsequently, their biological activity may be altered [10]. Oliveira et al. [11] found that there was a reduction in all phenolic compounds in both sorghum grain and flour within the first 60 days of storage, and that the retention of phenols ranged from 89.4% to 100% after 180 days of storage. Lang et al. [12] evaluated the influence of storage conditions on black rice phenolic compounds, showing an increase in total flavonoid content in the free form and a decrease in the bound part in grains stored under conventional and vacuum conditions, as well as illustrating a decrease in the total phenolics content regardless of the storage conditions. Whole wheat flour is more likely to be subject to rapid oxidization because lipids, lipase, and lipoxygenase are also contained in the germ and bran [10]. Few studies have focused on the phytochemical and biological activity losses in wheat flour during storage. This aspect necessitates further study.

The longevity of seeds depends on storage conditions such as seed moisture, oxygen, and temperature. The effects of oxygen are variable, depending on crop species and storage conditions. According to the study of Chiu et al. [13], partial vacuum storage could reduce ROS-mediated peroxidative damage during storage and improve the longevity of primed sweet corn (Zea mays L.) seeds. A paper bag is a normal mode to store wheat flour. Often, wheat flour must be stored for a few weeks in the kitchen. Little attention is paid to the longevity of the flour of seeds, especially the effect of oxygen in the storage atmosphere. In addition, due to the differences in wheat polyphenol content in different genotypes, it was important to select a cultivar with a

high nutritional potential. The objectives of the present work were to study the effect of storage in air and under vacuum conditions at different storage times on phenolic compounds and the antioxidant capacities of extracts from different wheat cultivars. Verna and Timilia were representative ancient wheat cultivars used in the present study, whereas Sagittario and Claudio were the representatives of modern varieties.

2. Materials and Methods

2.1. Chemicals and Reagents

Vanillic acid, *p*-hydroxybenzoic acid, ferulic acid, *p*-coumaric acid, sinapic acid, and syringic acid (used as standard) were purchased from Sigma-Aldrich (Milan, Italy). Methanol, acetic acid, and water (HPLC-grade, Lichrosolv®) were acquired from Merck (Darmstadt, Germany). All other chemicals and solvents were analytical grade.

2.2. Wheat Samples

Wheat samples consisted of two ancient (Verna and Timilia) and two modern (Sagittario and Claudio) Italian wheat cultivars, in which Verna and Sagittario were the representative common wheat (*Triticum aestivum* L.) genotypes and Timilia and Claudio were the representative durum wheat (*Triticum turgidum ssp. Durum*) genotypes, respectively. Grains were harvested from the same location in Italy (Argelato, latitude 44°39′57″ N, longitude 11°19′43″ E, 25 m a.s.l.) in the growing season 2018−2019 in organic agriculture. Whole wheat flour of each variety was obtained from a stone mill. The flour was stored in paper milling bags, commonly used for the storage of flour in aerated conditions, and plastic bags for vacuum conditions at an ambient temperature ranging from 25 to 30 °C. After milling, the flour was measured immediately at week 0. The analyses were repeated after different storage intervals (2, 4, and 8 weeks) to simulate a time range of home practice and to evaluate the effects of storage time and storage conditions on the phenolic content and antioxidant activity.

2.3. Extraction of Free and Bound Polyphenol

The extraction of free and bound phenolic compounds was performed as previously described by Dinelli et al. [14]. Whole wheat flours (1 g) were added to 10 mL of cold 80% ethanol (4 °C) to obtain the free soluble compounds, followed by further

hydrolysis extraction steps using sodium hydroxide and hydrochloric acid to release the bound fractions. The free and bound extracts were pooled together, evaporated to dryness, and reconstituted to a final volume of 4 mL in 70% ethanol. The extracts were filtered through a 0.45 µm PTFE membrane filter and stored at −20 °C until analysis.

2.4. Determination of Total Polyphenol Content

The TPC of extracts was determined according to the Folin–Ciocalteu method [15]. Briefly, 40 µL of diluted phenolic extract was mixed with 1.6 mL of distilled water and 100 µL of Folin–Ciocalteu reagent. After incubation for 5 min, 300 µL of aqueous sodium carbonate solution (0.2 g/mL) was added. The absorbance was measured at 765 nm after 2 h incubation in the dark. The TPC was expressed as mg of gallic acid equivalents (GAE) per 100 g of whole wheat flour.

2.5. Determination of Total Flavonoids Content

TFC was estimated using a colorimetric method described previously by Adom et al. [16]. Briefly, 250 µL of diluted phenolic extract was added to 1.25 mL of distilled water and 75 µL of sodium nitrite (5%) and then incubated for 6 min. There was a reaction with aluminum chloride (10%) to form a flavonoid-aluminum complex within 5 min of incubation. Thereafter, 0.5 mL of 1 M sodium hydroxide was added to stop the reaction. The absorbance was immediately measured at 510 nm. The TFC was expressed as mg catechin equivalents (CE) per 100 g of whole wheat flour.

2.6. HPLC-MS/MS Analysis

A Waters e2695 Alliance HPLC System combined with a Waters ACQUITY QDa Mass Detector were used for the phenolic profile analysis, as described by Truzzi [17]. A Luna Omega C-18 (250 × 4.6 mm, 5 µm) (Phenomenex, CA, USA) column was used with a temperature at 40 °C. The mobile phase was solvent A: water + 0.1% acetic acid and solvent B: methanol. A gradient experiment was conducted as follows: at 0 min, 20% solvent B; at 2 min, 30% solvent B; at 10 min, 38% solvent B; at 17 min, 20% solvent B. The flow rate and the injection volume were 1.2 mL/min and 10 µL, respectively. Single-ion recording (SIR) was used in the Electrospray Ionization ESI negative mode, with cone voltage (8 V), Probe (600 °C), sampling rate (10 points/sec), capillary (Pos: 0.8, Neg:0.8). The monitored (M–H)-ions were m/z 136.88, 162.93,

167.00, 192.93, 196.94, and 223.09 for *p*-hydroxybenzoic acid, *p*-coumaric acid, vanillic acid, ferulic acid, syringic acid, and sinapic acid, respectively. Data acquisition and instrument control were performed using the Empower 3 software.

2.7. Assay of Antioxidant Activity in Vitro

2.7.1. Assay of DPPH Radical Scavenging Activity

The DPPH assay was performed following a modified procedure reported by Floegel et al. [18]. Briefly, 1 mM of DPPH solution in 80% (v/v) methanol was prepared, for which of the absorbance was adjusted to 0.650 ± 0.020 at 517 nm. Diluted phenolic extract (50 µL) was added to 2.95 mL of DPPH solution. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. The results were expressed as µmol of Trolox equivalents (TE) per g of whole wheat flour.

2.7.2. Assay of Ferric Reducing Antioxidant Potential (FRAP)

The FRAP assay was conducted according to Benzie and Strain [19], with some modifications. The freshly prepared FRAP working solution (WS) consisted of 300 mM of acetate buffer pH 3.6, 10 mM of TPTZ, and 20 mM of ferric chloride (10:1:1, v:v:v). A volume of 80 µL of diluted (1:1, v/v) phenolic extracts was added to 2.4 mL of WS and incubated for 1 h in the dark. The absorbance was measured at 593 nm. The results were expressed as mmol Fe^{2+} per 100 g of whole wheat flour.

2.8. Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) test with the software SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was considered at $p < 0.05$. Results were expressed as the mean ± SD.

3. Results

3.1. Moisture Content

Water is an important composition of food, influencing the physical, chemical, and biological characteristics of food. The stability and shelf life of food is also affected by the water content. Water can enhance or weaken chemical reactions due to its effect on molecular mobility [20]. Since moisture content indicates the residual water in flours, this parameter is a crucial property of flour products. In the present study, the moisture

content of the wheat flours was at a satisfactory level (ranging from 11.99% to 12.49%) at Week 0, as shown in Table 1. The flours of the four cultivars under vacuum storage had a stable moisture content over 8 weeks of storage. Regarding the flour maintained in the air, the moisture content decreased at Week 2 compared with freshly prepared flour. However, there was an increase in moisture content from Week 4 to 8.

Table 1. The moisture content of four wheat genotypes stored under different conditions and for different lengths of time.

Cultivar	Storage Condition	Storage Time			
		Week 0	Week 2	Week 4	Week 8
Verna	Air	12.49 ± 0.29%aA	11.04 ± 0.11%cB	10.61 ± 0.35%cdB	12.90 ± 0.13%aA
	Vacuum	12.49 ± 0.29%aA	12.35 ± 0.08%aAB	11.96 ± 0.33%aB	12.07 ± 0.10%bAB
Timilia	Air	11.99 ± 0.20%bB	10.84 ± 0.00%cC	10.71 ± 0.17%cC	12.69 ± 0.19%aA
	Vacuum	11.99 ± 0.20%bA	11.76 ± 0.15%bAB	11.57 ± 0.16%abAB	11.53 ± 0.32%cB
Sagittario	Air	12.06 ± 0.16%bB	10.97 ± 0.27%cC	10.53 ± 0.40%cdC	12.78 ± 0.21%aA
	Vacuum	12.06 ± 0.16%bA	11.61 ± 0.44%bA	11.45 ± 0.16%bA	11.98 ± 0.39%bA
Claudio	Air	11.99 ± 0.20%bB	10.89 ± 0.18%cC	10.18 ± 0.17%dC	12.51 ± 0.04%aA
	Vacuum	11.99 ± 0.20%bA	11.65 ± 0.28%bAB	11.31 ± 0.29%bB	11.57 ± 0.22%cAB

Data are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences in values at $p < 0.05$. Values in the rows with different capital letters are significantly different at $p < 0.05$.

3.2. Total Polyphenol Content

The TPC of freshly prepared whole wheat flour from the four cultivars ranged from 241.32 to 283.72 GAE mg/100 g (represented as Week 0 in Table 2). Verna exhibited a significantly higher phenolic content than the remaining three cultivars.

Table 2. Total polyphenol contents expressed as gallic acid equivalents of four wheat cultivars (mg/100 g) stored under different conditions and lengths of time.

Cultivar	Storage Condition	Storage Time			
		Week 0	Week 2	Week 4	Week 8
Verna	Air	283.72 ± 5.18aA	282.72 ± 8.31aA	258.01 ± 4.74aB	236.06 ± 6.13bC
	Vacuum	283.72 ± 5.18aA	282.82 ± 4.94aA	260.41 ± 7.74aB	247.15 ± 4.13aC
Timilia	Air	273.41 ± 4.29aA	271.21 ± 17.51aA	233.83 ± 3.38bB	228.61 ± 1.03cB
	Vacuum	273.41 ± 4.29aA	272.43 ± 6.90aA	239.08 ± 3.70bB	219.89 ± 6.64dB
Sagittario	Air	244.52 ± 7.38bA	211.33 ± 6.86bBC	216.50 ± 7.24cB	199.74 ± 3.22dC
	Vacuum	244.52 ± 7.38bA	217.32 ± 3.19bB	215.88 ± 2.17cB	200.75 ± 2.13dC
Claudio	Air	241.32 ± 4.85bA	216.20 ± 4.19bB	210.63 ± 5.70cdB	191.53 ± 1.20eC
	Vacuum	241.32 ± 4.85bA	209.37 ± 4.11bB	204.23 ± 4.29dB	190.93 ± 3.08eC

Data are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences in values at $p < 0.05$. Values in the rows with different capital letters are significantly different at $p < 0.05$.

The results illustrated that the TPC of the four cultivars was affected by the storage

time. Overall, the TPC decreased significantly over the 8 weeks in all cultivars. Verna still showed the highest level of TPC. The rankings of the cultivars in order of increasing losses of TPC between Week 0 and Week 8 of storage were Timilia < Verna < Sagittario < Claudio for aerated storage and Verna < Sagittario < Timilia < Claudio for vacuum storage (with the corresponding percentages losses at 16.39, 16.80, 18.31, 20.63, and 12.89, 17.90, 19.57, 20.88, respectively). There were no significant differences in TPC ($p < 0.05$) for the four cultivars between the air and vacuum storage conditions within the first 4 weeks. However, there was a significant difference in TPC between the air and vacuum storage conditions for Verna and Timilia at Week 8.

3.3. Total Flavonoid Content

In freshly prepared flour (represented as Week 0 in Table 3), the TFC of the four cultivars varied from 62.82 to 79.13 CE mg/100 g. Verna had the highest flavonoid content among the four cultivars.

After 8 weeks, the TFC of all the cultivars decreased significantly when compared to the freshly prepared flour. Verna also showed the highest level of TFC. The TFC in Claudio decreased up to 31.18%. However, there was a sharp increase from Week 4 to Week 8 for all cultivars. There were no significant differences in TFC between the air and vacuum storage conditions, with the exception of Claudio at Week 4 and Week 8 and Sagittario at Week 8.

Table 3. Total flavonoid contents expressed as catechin equivalents of four wheat cultivars (mg/100 g) stored under different conditions and for different lengths of time.

Cultivar	**Storage Condition**	**Storage Time**			
		Week 0	**Week 2**	**Week 4**	**Week 8**
Verna	Air	79.13 ± 3.63aA	58.72 ± 2.17aC	52.23 ± 0.74aD	67.36 ± 1.37aB
	Vacuum	79.13 ± 3.63aA	58.49 ± 3.91aC	52.98 ± 1.01aC	67.87 ± 2.33aB
Timilia	Air	69.09 ± 1.31bA	51.20 ± 5.82abcBC	45.74 ± 1.09cC	54.71 ± 1.53cB
	Vacuum	69.09 ± 1.31bA	48.94 ± 4.87bcBC	44.89 ± 1.74cC	52.68 ± 0.93cB
Sagittario	Air	71.24 ± 1.68bA	53.34 ± 5.72abC	48.36 ± 0.83bC	61.21 ± 1.76bB
	Vacuum	71.24 ± 1.68bA	51.20 ± 4.22abcB	48.23 ± 0.23bB	52.32 ± 3.36cB
Claudio	Air	62.82 ± 1.57cA	45.47 ± 0.97cB	42.98 ± 1.08dC	47.24 ± 0.32dB
	Vacuum	62.82 ± 1.57cA	43.78 ± 0.08cB	40.76 ± 0.46eC	43.23 ± 2.51eBC

Data are expressed as mean ± SD (n = 3). Different lowercase letters indicate significant differences in values at $p < 0.05$. Values in the rows with different capital letters are significantly different at $p < 0.05$.

3.4. Analysis of the Phenolic acid Profiles

Six phenolic acids, including *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, were all found in the wheat flour of the four cultivars under the different storage conditions and lengths of time. Table 4 showed that the most abundant phenolic acid in the four cultivars was ferulic acid, which showed the highest mean value of 355.55 µg/g. This was followed by *p*-coumaric acid, with a mean content of 56.35 µg/g at Week 0 for the four cultivars.

The amount of phenolic acid, *p*-hydroxybenzoic acid, decreased significantly after 8 weeks of storage in all cultivars, except in Sagittario. The least reduction in *p*-hydroxybenzoic acid was shown in Verna stored in air, which decreased to 8.67%. *p*-coumaric acid also decreased over storage for 8 weeks, except in Sagittario, which was stored under vacuum. In Verna, *p*-coumaric acid was very unstable and was shown to decrease up to 34.13% over 8 weeks. Decreases in ferulic acid were shown in Verna, Timilia, and Claudio, but more than 88.93% remained after 8 weeks. Meanwhile, there was an increase in Sagittario after storage for 8 weeks with respect to ferulic acid. The losses of sinapic acid were significantly found in all cultivars from freshly prepared flour to the 8th week, except Claudio. The biggest loss of sinapic acid was shown in Timilia stored in air, which was up to 31.61% over the 8-week period. On the contrary, an increase was shown in vanillic acid and syringic acid from Week 0 to Week 8 in all the other three cultivars, with the exception of Claudio. In particular, an increase in the syringic acid up to 297.03% was detected in Sagittatio under vacuum storage for 8 weeks. The concentration of individual phenolic acids was shown to decline or increase at different rates, possibly due to their chemical structure, biochemical pathway relationship, and storage conditions.

Table 4. Phenolic acid profiles of four wheat varieties cultivars (μg/g) stored under different conditions and for different lengths of time.

Cultivar	Storage Time	Storage Condition	Phenolic Compounds					
			p-Hydroxybenzoic Acid	Vanillic Acid	Syringic Acid	*p*-Coumaric Acid	Ferulic Acid	Sinapic Acid
Verna	Week 0		18.76 ± 0.05a	23.50 ± 0.21c	13.13 ± 0.96h	92.79 ± 4.08a	421.25 ± 9.39a	19.95 ± 1.48a
	Week2	Air	18.57 ± 0.13b	16.80 ± 0.09h	7.32 ± 0.36mno	65.75 ± 4.14c	356.28 ± 13.22cd	12.93 ± 1.28ef
		Vacuum	17.29 ± 0.07d	17.27 ± 0.15h	7.94 ± 0.64m	57.46 ± 4.12e	373.77 ± 9.55bc	17.61 ± 1.64b
	Week 4	Air	18.29 ± 0.15c	28.73 ± 0.09a	19.32 ± 0.41c	70.45 ± 4.42b	423.44 ± 13.09a	17.03 ± 1.02b
		Vacuum	17.1 ± 0.05e	23.73 ± 0.72c	14.04 ± 0.39fg	66.73 ± 2.86bc	354.28 ± 14.51cd	13.87 ± 0.56de
	Week 8	Air	17.13 ± 0.03de	24.72 ± 0.17b	13.98 ± 0.22fg	63.29 ± 2.59cd	386.04 ± 8.90b	15.53 ± 1.19c
		Vacuum	16.54 ± 0.06f	23.70 ± 0.46c	14.39 ± 0.40ef	61.12 ± 1.77de	393.23 ± 18.92b	15.26 ± 1.58c
Timilia	Week 0		13.52 ± 0.08g	21.94 ± 0.40d	11.81 ± 0.24ij	47.94 ± 2.66g	343.60 ± 17.88d	14.53 ± 0.50cd
	Week 2	Air	11.95 ± 0.07j	20.70 ± 0.72f	10.73 ± 0.48k	39.12 ± 1.93i	313.79 ± 8.15e	8.57 ± 0.44kl
		Vacuum	10.13 ± 0.06m	12.83 ± 0.23j	5.76 ± 0.27q	37.05 ± 1.79ij	307.89 ± 3.41ef	8.10 ± 0.16l
	Week 4	Air	11.94 ± 0.15j	25.55 ± 0.34b	16.95 ± 0.36d	44.83 ± 3.24gh	374.18 ± 8.85bc	9.62 ± 0.47jk
		Vacuum	10.78 ± 0.14l	21.81 ± 0.21de	13.52 ± 0.46gh	38.35 ± 1.86i	293.08 ± 13.81efg	7.29 ± 0.25l
	Week 8	Air	11.77 ± 0.13jk	22.31 ± 0.41d	12.37 ± 0.40i	37.23 ± 2.80ij	311.91 ± 11.73e	9.93 ± 0.25hijk
		Vacuum	11.73 ± 0.22k	23.80 ± 0.49c	14.95 ± 0.42e	40.54 ± 2.57hi	353.50 ± 17.37cd	11.28 ± 0.63gh
Sagittario	Week 0		12.63 ± 0.09h	11.95 ± 0.37j	7.39 ± 0.55mn	46.32 ± 1.27g	350.32 ± 13.05d	15.50 ± 0.50c
	Week 2	Air	12.21 ± 0.05i	14.93 ± 0.13i	11.24 ± 0.37jk	40.84 ± 1.65hi	340.77 ± 15.67d	10.02 ± 0.46hijk
		Vacuum	4.54 ± 0.05s	4.55 ± 0.07m	6.56 ± 0.66op	33.75 ± 2.03jk	314.41 ± 5.91e	0.77 ± 0.03m
	Week 4	Air	11.94 ± 0.12j	12.64 ± 0.56j	7.15 ± 0.27mno	44.95 ± 1.52gh	311.66 ± 10.87e	10.22 ± 0.84hij
		Vacuum	11.82 ± 0.11jk	21.85 ± 0.19de	21.42 ± 0.57ab	40.30 ± 2.31i	351.91 ± 14.21cd	9.75 ± 0.16jkl
	Week 8	Air	12.64 ± 0.06h	21.80 ± 0.27de	20.98 ± 0.41b	44.99 ± 1.42gh	389.95 ± 13.53b	13.34 ± 0.79def
		Vacuum	12.54 ± 0.06h	21.84 ± 1.32de	21.96 ± 0.24a	52.06 ± 0.81f	373.59 ± 13.80bc	12.43 ± 0.64fg
Claudio	Week 0		10.21 ± 0.04m	18.20 ± 0.99g	5.98 ± 0.24pq	38.33 ± 1.67i	307.04 ± 4.13ef	9.85 ± 0.28hijk
	Week 2	Air	7.14 ± 0.05q	10.93 ± 0.35k	3.80 ± 0.12r	33.22 ± 1.40jkl	290.56 ± 17.61efg	8.23 ± 0.39l
		Vacuum	6.56 ± 0.09r	9.09 ± 0.79l	3.32 ± 0.13s	24.75 ± 1.58n	287.65 ± 7.19fg	11.34 ± 0.94gh
	Week 4	Air	8.58 ± 0.10n	20.89 ± 0.98ef	9.65 ± 0.39l	37.60 ± 2.95ij	336.73 ± 12.61d	9.74 ± 0.44jkl
		Vacuum	7.36 ± 0.09p	18.48 ± 0.70g	7.85 ± 0.42m	28.33 ± 0.75mn	304.35 ± 5.71ef	9.76 ± 0.28jkl
	Week 8	Air	8.42 ± 0.06n	16.65 ± 0.39h	6.69 ± 0.37nop	29.32 ± 1.24lm	299.11 ± 14.14ef	11.15 ± 0.67ghi
		Vacuum	7.86 ± 0.14o	17.20 ± 0.77h	7.34 ± 0.48mno	31.71 ± 1.84klm	273.04 ± 12.24g	9.97 ± 0.54hijk

Data are expressed as mean ± SD (n = 3). Values in the column with different letters are significantly different at $p < 0.05$.

3.5. Antioxidant Activity

3.5.1. DPPH Assay

The initial antioxidant activity values of the four cultivars determined by the DPPH assay ranged from 6.78 to 7.87 μmol trolox/g. Verna showed the best antioxidant activity compared to the remaining cultivars (Figure 1).

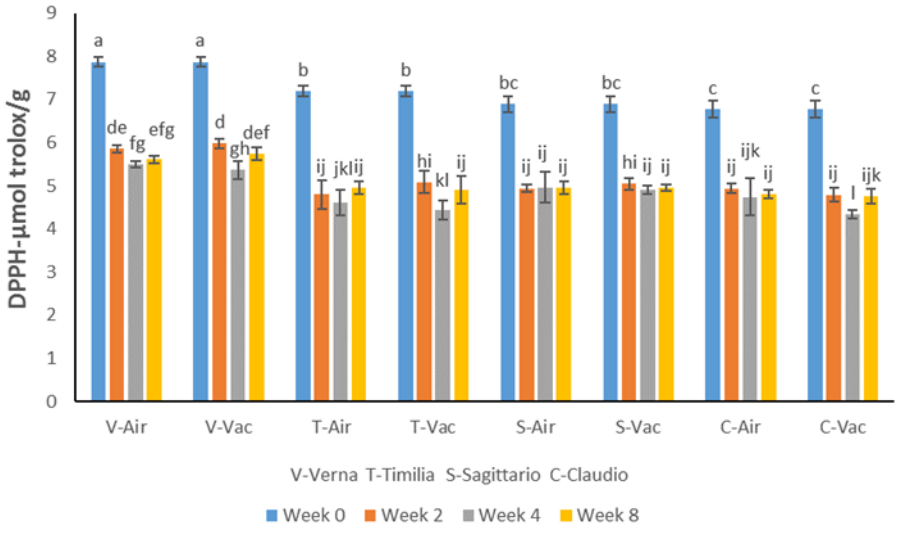

Figure 1. Antioxidant activity shown by DPPH assay of four wheat cultivars storage in different conditions and for different lengths of time. Different letters show significant differences at $p < 0.05$.

The antioxidant activity shown by the DPPH assay of the four cultivars decreased significantly after 8 weeks of storage. However, there was no significant difference between the air and vacuum storage conditions regarding the DPPH values. In air, the decreases were 28.72, 31.25, 28.16, and 29.20% in Verna, Timilia, Sagittario, and Claudio, respectively, over the 8-week period. Regarding the vacuum condition, the decrease was 27.06, 31.94, 28.16, and 29.79% in the four cultivars, respectively. The DPPH antioxidant activity in Verna storage under vacuum decreased the least among the four cultivars. Approximately 72.94% of the antioxidant activity was maintained in Verna after the 8-week storage period.

3.5.2. FRAP Assay

The initial antioxidant activity values of the four cultivars determined by the FRAP assay ranged from 2.18 to 2.57 mmol Fe^{2+}/100 g. Verna showed a higher antioxidant activity than the other three cultivars, as represented by the FRAP assay (Figure 2).

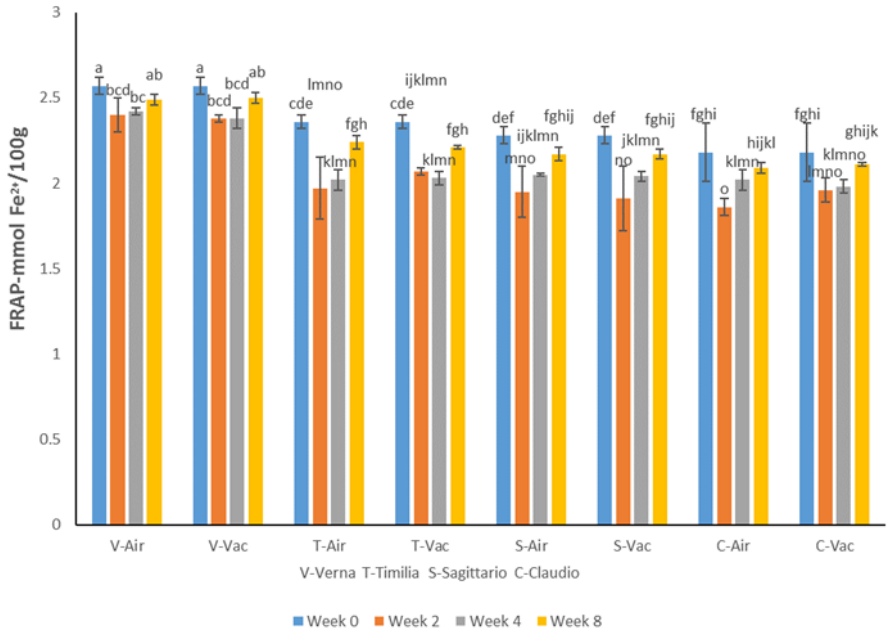

Figure 2. Antioxidant activity shown by the FRAP assay of four wheat cultivars storage in different conditions for different lengths of time. Different letters show significant difference at $p < 0.05$.

The ability of wheat polyphenol to reduce Fe^{3+} to Fe^{2+} was not significantly affected by either storage conditions or time over the 8-week storage period for all the four cultivars. There was a reduction in antioxidant activity after Week 2 but a recovery at the end of the storage period.

4. Discussion

In the present study, we investigated the moisture content, phenolic acids profile, TPC, TFC, and AA of the whole wheat flour extracts of four cultivars, Verna, Timilia, Sagittario, and Claudio, from same harvest year and same farm in Italy. Different cultivars of wheat showed different levels of TPC, TFC, and AA as well as phenolic acids profile during the entire storage period as well as under the two storage conditions. The wheat polyphenol was influenced more by storage time (0, 2, 4, 8 weeks) than storage conditions (aerated and under vacuum).

4.1. Moisture Content

The flours of the four cultivars under vacuum storage had a stable moisture content without contact with water and oxygen. However, the moisture content decreased from week 0 to week 4 for the four cultivars stored in the air, and then there was an increase

from the 4th week to the 8th week. There was also an increase observed in the TFC and AA, as determined by the DPPH assay in the same period. This indicated that moisture content is a key factor affecting the stability of some phenolic compounds during storage.

4.2. Total Polyphenol and Antioxidant Activity

As reported by Silvestro [4], wheat cultivar affected the TPC of bound forms and the AA determined by FRAP. Differences in TPC and AA among the four cultivars of wheat were also observed in our study. Verna exhibited a higher amount of TPC, TFC, and AA than Timilia, Sagittario, and Claudio in freshly prepared flour under the two storage conditions. Timilia was characterized by the second highest total amount of TPC. Regarding TFC, Timilia and Sagittario were significantly higher than Claudio. With respect to AA, there was no significant difference among Timilia, Sagittario, and Claudio in freshly prepared flour.

The results of our study showed that storage time significantly influenced the phenolic contents in whole wheat flour. After 8 weeks of storage, the TPC, TFC, and AA of all the cultivars decreased when compared to the freshly prepared flour. This may be attributed to the oxidation of polyphenols [10]. Similar results were reported by Oliveira et al. [11], who showed a reduction in sorghum flour after 60 days of storage. Verna was characterized by smaller losses of TPC and TFC than the other three cultivars over 8 weeks. In addition, Verna showed the highest retention of AA represented in DPPH among four cultivars of about 73%. With respect to the TPC and TFC, the biggest reduction was found in Claudio compared to the freshly prepared flour. Regarding the AA represented in DPPH, Timilia showed a lower retention than other three cultivars over 8 weeks. In fact, a sharp increase in TFC from the 4^{th} to the 8^{th} week was reported in this study. It is feasible that unstable glycosides may have been transformed, resulting in an increase in TFC. Similarly, AA represented by the DPPH assay resulted in a significant increase during the same period. It seems that the decrease in AA did not follow the reduction in the polyphenol content of whole wheat flour. In similar findings in the research of Patthamakanokporn et al. [21], a continuous decrease in TPC was found in homogenized guava, but the FRAP antioxidant activity did not decrease during

the 3-month storage. A possible reason for this was that the antioxidant activity was not related to an individual phenolic or classes of phenolics but to a synergistic effect between different classes of polyphenol.

However, the AA determined by the FRAP assay was different with the DPPH assay. Different antioxidants have different mechanisms against free radicals [22], and probably different polyphenol molecules in wheat were responsible for the differences in FRAP and DPPH. Liu et al. [23] found that the total phenolic contents showed a higher correlation with antioxidant capacity when using FRAP assays than when using the DPPH assay among the fruits and vegetables analyzed. A reduction was shown at the 2^{nd} week but a recovery was shown at the 8^{th} week in the FRAP assay in our study. In fact, the decreases in the total polyphenol contents were not always influenced by AA [20]; the oxidation of polyphenol was expected to result in a lower antioxidant capacity, but the formation of new antioxidant polymers or degradation products of phenolics also had AA [24]. In addition, the phenolic profile may be changed during storage, as some phenolics could be degraded while other new phenolics could be formed [25]. This could also increase the TPC and compensate for the loss of AA from the degraded compounds. According to Flores et al. [26], an improvement in TPC and a slight increase in AA in spray-dried blueberry pomace extract were observed after a 40-day storage. It is possible to explain this by some phenolic compounds being newly produced during the storage time. Some reports have shown that anthocyanins could decompose to benzoic acid derivatives such as syringic acid or 4-hydroxybenzoic acid [27,28].

Vacuum conditions were supposed to be stable without contact with water and oxygen. However, the results were different from the hypothesis, as most of the mean values for the assessed parameters except for the moisture content of the four cultivars were not able to significantly discriminate between storage in air and under vacuum in our research, which was similar to the results reported by Ghirardello et al. [25]. The latter were not able to detect significant difference among storage conditions (ambient air, vacuum, or modified atmosphere) regarding the loss of TPC and AA in hazelnuts

(Corylus avellana L.) after 12 months of storage.

4.3. Phenolic Acids Profile

Ferulic acid and *p*-coumaric acid were the principle phenolic acid components of the four cultivars, as reported previously by Loreto et al. [5]. The highest ferulic acid content was reported for Verna, which is lower than that in the study of Gottia et al. [7]. The ferulic acid content from Claudio was higher than that reported by Loreto et al. [5].These differences may be due to the difference in harvest year, storage conditions, and other environmental factors.

To the best of our knowledge, no data are available regarding the effect of different storage times and conditions on changes in the phenolic acid profiles of whole wheat flour. The storage time influenced the concentration of each phenolic acid, but it was difficult to compare within different storage conditions in our study. Sinapic acid and *p*-coumaric acid were significantly decreased in most of the wheat flours over the 8-week storage period. However, there was an increase in vanillic acid and syringic acid during this period. Differences could possibly be explained by the different rates of degradation and/or synthesis of each phenolic acid [25]. Different phenolic acids have different chemical structures and also present different structures in different fractions, such as free and bound phenolic fractions. Structural difference plays an important role in individual reducing capacity [29]. The storage process changes the content of phenolic acids in the plant material and decreases or increases in their content were both observed [30]. Zhou et al. [29] found a reduction in the concentration of ferulic acids from brown rice after storage for 6 months. According to Bolling et al. [31], there were dynamic metabolic processes affecting phenolic acid content during storage, including an increase in polyphenol extractability; the degradation of polymeric polyphenols; and, consequently, an increase in soluble phenolics during storage. In addition, this was possibly attributable to a biosynthesis pathway of these six phenolic acids in plants [5,7]. For example, *p*-coumaric acid could be converted to ferulic acid and p-hydroxybenzoic acid, which could continue converting to vanillic acid. Similarly, ferulic acid could convert to sinapic acid and then syringic acid.

5. Conclusions

Most of the analyzed parameters illustrated differences in cultivars. Verna showed higher levels of TPC, TFC, and AA than Timilia, Sagittario, and Claudio during the entire storage period as well as under the two storage conditions. The phenolic content of whole wheat flour was not affected by the storage conditions-in fact, no significant differences between air and vacuum conditions were detected. Some reductions in total polyphenol were observed after storage for 8 weeks. The moisture content, flavonoid content, and antioxidant potential were decreased after 8 weeks of storage compared to freshly prepared flour, but a small increase between 4^{th} and 8^{th} week was also detected. Some changes, either decrease or increase in the concentration regarding the phenolic acids, were shown, and no compound was completely degraded during storage. Overall, our results firstly illustrated that the storage time significantly influenced the polyphenol content and profile, but we were not able to significantly discriminate between storage in air and under vacuum. Further knowledge in this area will be useful in determining the proper length and method of storage as well as the selection of cultivar in order for whole wheat flour to retain its bioactive compounds for benefits when consumed in daily life.

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Chapter 3 - Effect of Light Condition on Pigments, Phenolic Content, Antioxidant and Bioprotective Activities of Wheat Sprouts and Microgreens

Abstract

Wheat sprouts and microgreens constitute a good source of bioactive compounds, which are usually cultivated in a controlled environment with artificial light. In this study, wheat sprouts and microgreens obtained from four Italian wheat cultivars, including two ancient (Verna and Timilia) and two modern (Sagittario and Claudio) cultivars, grown under LED and fluorescent light for 6 days after 2 days germination under dark were evaluated for growth parameters, pigment and polyphenol contents, antioxidant and bioprotective activities. The results showed that the effect of light was generally not significant for wheat sprouts, much greater and species-specific for wheat microgreens. LED light failed to increase shoot length and fresh weight but increase the dry matter percentage of wheat microgreens. Verna microgreens grown under FL light showed the highest shoot length among all the cultivars selected. LED light significantly increased the Chlorophyll a, carotenoid, total phenolic and total flavonoid contents, as well as improved the antioxidant activities shown in the FRAP assay. Verna microgreens grown under LED light showed the highest pigment contents and Claudio microgreens grown under LED exhibited the highest TPC and antioxidant properties among the four cultivars. All the extracts of wheat microgreens exhibited bioprotective effects on Caco-2 cell viability, in which Sagittario grown under LED light demonstrated a better potential than the other cultivars. These results indicate that LED light should be recommended as a light source for production of wheat microgreens.

Keywords: Wheat sprouts and microgreens, LED and fluorescent light, pigment, polyphenol, antioxidant activity

1. Introduction

Wheat (*Triticum aestivum*) is one of the most important food cultivated worldwide. It is well known that wheat seeds possess health-promoting benefits due to the presence of fiber and various bioactive compounds [1]. However, the plant with different stage of growth (seeds, sprouts or microgreens), days after germination or height show a different bioactive profile and activity [2]. Recently an increasing interest has been directed to sprouts and microgreens which contain several phytochemicals used in food supplementation or pharmaceutics due to the health consciousness among consumers [3].

Cereal sprouts are the young seedlings, which are harvested just after seed gemination. They are always collected and eaten with whole parts, including the seeds [3,4]. Cereal spouts are reported with increased nutritional value compared to the seeds [5]. The total phenolic content of wheat sprouts increased after germination for 2 or 3 days in comparison with seeds, which was affected by germination temperature and time, as well as wheat genotype [6]. Wheat sprouts are new source of functional foods consumed normally in the form of tea [7] and sprouts powder could be added in the conventional flours [8].

The cereal microgreens are the seedlings obtained right after the emergence of true leave, which are few days old with 1 to 3 inch length [3,9]. Wheat microgreens, known as wheatgrass, are usually used to prepare fresh drinking juice or freeze-dried powder products with health benefits attributed to their bioactive compounds including chlorophylls, carotenoids, phenolics, flavonoids, vitamins, minerals and other antioxidants [10]. Chlorophyll is a key component of wheatgrass and has been reported as dietary supplement for chemotherapy due to the structural similarity to hemoglobin and bilirubin [11]. The phenolic compounds of wheatgrass, which are largely reported with antioxidant and health promoting properties [12]. Therefore wheatgrass has been found many benefits, including supportive treatment of thalassemia, ulcerative colitis, diabetes, hematological diseases and breast cancer [1].

Sprouts and microgreens are ideal for production under home-scale or area-limited

conditions due to their short growth period and high crop efficiency. They are perfect for the support of space life as they can be implemented without soil, or shallow substrates with few nutrients, and having a good performance in microgravity hydroponic system with low O_2 and nutrient solubility. In addition, containerized production of sprouts and microgreens provide an opportunity to be served freshly in salads, sandwiches, or used as an edible garnish by end-consumers [13]. Sprouts and microgreens are normally cultivated in a controlled environment with artificial light sources such as fluorescent (FL), high-pressure sodium and light emitting diode (LED) lamps [14]. FL lights are normal light sources in growth chambers, which could provide sustained photosynthetic photon fluence for high productivity. While FL lights show low efficiency for energy conversion to light with limited photon output, and have short lifespan [4]. LED is a cheap and environmentally friendly source of light, which can provide uniform light distribution [15]. Compared to conventional lamps, LED lamps could provide high light intensity with low heat emission and spectral quality with selective excitation of photoreceptors including phytochrome and cryptochrome and optimize production of phytochemicals [16]. It was reported that the total content of polyphenol, flavonoid and phenolic acids generally increased after the treatment with blue LED light for einkorn and red LED light for emmer wheatgrass [3]. The pigment content in plants is very sensitive to light spectra and intensity thus their content can be increased or decreased by slight differences in the light. It was shown that pigment content increased for einkorn wheatgrass grown under a treatment for combinations of blue and red LED light (blue $\geq$ 50%) [17].

However, the research on the effect of LED and FL light on wheat sprouts and microgreens is limited. In addition, it is also important to select a cultivar with high nutrient contents to produce wheat sprouts and microgreens as different amounts of bioactive content present in various wheat cultivars. Therefore, the objective of the present work was to study the effect of LED and FL light conditions on the growth and nutritional value of sprouts and microgreens from different wheat cultivars and to find if the effects were related to the wheat cultivar specific properties. To this aim, four

cultivars were chosen: Verna and Timilia were representative ancient wheat cultivars used in the present study, whereas Sagittario and Claudio were the representatives of modern cultivars.

2. Materials and Methods

2.1. Materials

Seeds from four cultivars of wheat, including two ancient (Verna and Timilia) and two modern (Sagittario and Claudio) Italian wheat cultivars were harvested from the same location in Italy (Argelato, latitude 44°39′57″N, longitude 11°19′43″E, 25 m a.s.l.) in the growing season 2018 − 2019 in organic agriculture, in which Verna and Sagittario were the representative common wheat (*Triticum aestivum* L.) genotypes and Timilia and Claudio were the representative durum wheat (*Triticum turgidum ssp.* Durum).

2.2. Growing Conditions

Seeds were soaked in 0.5% aqueous sodium hypochlorite for 15 min at room temperature for sterilization and rinsed with tap water and distilled water 3 times respectively. Then seeds were soaked in distilled water overnight (seed : H_2O = 1:10) and drained. 25 seeds were sown in each plastic pot (13 × 20 × 8 cm) filled with peat substrate (pH 6.5, electricity conductivity 0.50 dS/m, porosity 90% v/v) and germinated in the dark for 48 h.

Sprouts and microgreens were grown under LED panel units (Top light, Natural Indoor Plus, 230 V, 75W, C-LED, Imola, Italy) with an emission wavelength ranging 400 – 700 nm: blue (400 – 500 nm), green (500 – 600 nm) and red (600 – 700 nm) and exposed to a homogeneous photosynthetic photon flux density (PPFD) of 270 μmol m^{-2} s^{-1}. And FL light (FH21830, 21 W, warm white 830, 85 cm, OSRAM SpA, Milan, Italy) was used as control with PPFD of 65 μmol m^{-2} s^{-1}. Each lighting treatment contained five replicate pots for each cultivar. Pots were arranged randomly and 85 cm away from the lamps and systematically rotated every day to improve the uniformity of the light environment.

The plants were grown in a growth chamber at 18 ± 2°C, photoperiod 16/10 h day/night, and relative humidity of 72% – 83%. The seed pots were sprayed daily with

tap water, as needed. After the lighting experiment, sprouts and microgreens were harvested from light treatment on Day 0, 1 and Day 4, 5, 6 around 9:00 am. Wheat sprouts including shoot, seed coat and root were harvested at Day 0 and 1, while microgreens were taken from 1 cm above the pot, only the shoot, for all the analysis of total extracts.

2.3. Growth Parameter

Fresh weight was weighed immediately after harvest. Sprouts and microgreens were oven dried to constant weight at 80 °C for determining plant dry weight. Shoot length were measured with 25 individuals per each replicate (3 replicates) every harvest day.

2.4. Shoot Pigments (Chlorophyll and Carotenoid) Analysis

Pigments extraction was performed at the same day of harvest according to a previous study with some modifications [18]. Sprouts and wheatgrass were extracted with 80% (v/v) acetone-water (samples: solvents = 1:10 (w/v)) and then vortexed for 5 min. The homogenized tissues were centrifuged (7000 rpm) for 3 min, and the supernatants were collected. The whole extraction processes were repeated until the pellets were discolored. All the supernatants were filtered through Whatman No. 1 filter paper and analyzed with UV-VIS spectrophotometer at 470, 648, and 663 nm wavelengths. The concentration was calculated using the equations as follows:

Chlorophyll a (μg/mL) = $12.25 \times A_{663} - 2.55 \times A_{648}$

Chlorophyll b (μg/mL) = $20.31 \times A_{648} - 4.91 \times A_{663}$

Total chlorophylls (μg/mL) = $17.76 \times A_{648} + 7.34 \times A_{663}$ (Chlorophyll a + Chlorophyll b)

Total carotenoids (μg/mL) = $(1000 \times A_{470} - 1.82 \times$ (Chlorophyll a) $- 85.02 \times$ (Chlorophyll b))/198

(The A_{663}, A_{648}, A_{470} represent the absorbance at 663,648,470 nm)

2.5. Total Aqueous Extraction

Wheat sprouts and microgreens were extracted according to a previously reported method [19]. Briefly, samples were ground with a mortar and pestle with water (1:10, w/v, samples: H_2O) on the ice. The mixture was transferred into a beaker and stirred for 20 − 30 minutes, and then centrifuged at 4000 rpm for 5 minutes. The supernatant was

removed and filtered with 0.45 μm filter membrane, and the liquid sample was maintained at −20℃ for further analysis.

2.5.1. Determination of Total Phenolic Content

The total phenolic content (TPC) of extracts was analyzed using the Folin-Ciocalteu method [3] with tiny modifications. To summarize, 40 μL total extract was added to 1.6 mL of distilled water and 100 μL of Folin-Ciocalteu reagent and then incubated for 5 min, followed by 300 μL of aqueous sodium carbonate solution (0.2 g/mL). Then, after remaining in the dark for 2 h at room temperature, the absorbance was determined at 765 nm. The results were expressed as mg of gallic acid equivalents (GAE) per 100 g fresh weight (FW) of samples.

2.5.2. Determination of Total Flavonoid Content

The total flavonoid content (TFC) was measured using a colorimetric method [20] with some modifications. In brief, 250 μL of total extract was mixed with 1.25 mL of distilled water and 75 μL of sodium nitrite (5%) and then incubated for 6 min. The mixture was allowed to react with 150 μL of aluminum chloride (10%) to form a flavonoid-aluminum complex within 5 min of incubation. Thereafter, the reaction stopped by mixing with 0.5 mL of sodium hydroxide (1 M). The absorbance was immediately determined at 510 nm. The results were expressed as mg catechin equivalents (CE) per 100 g fresh weight.

2.5.3. Determination of Radical-scavenging Activity

The assay was conducted according to a modified method [21]. In brief, 1 mM of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in 80% (v/v) methanol was prepared firstly. Then, absorbance of DPPH solution was adjusted to 0.650 ± 0.020 at 517 nm using 80% (v/v) methanol. Total extract (50 μL) was mixed with 2.95 mL of DPPH solution and incubated for 30 min at room temperature in the dark. Then the absorbance was determined at 517 nm. The results were expressed as μmol Trolox equivalents (TE) per g fresh weight of samples.

2.5.4. Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was performed following previous method [22] with minor

modifications. The fresh FRAP working solution was prepared with reagents as follows: 300 mM of acetate buffer pH 3.6, 10 mM of 2,3,5-Triphenyltetrazolium chloride (TPTZ), and 20 mM of ferric chloride (10:1:1, v:v:v). A volume of 80 µL of diluted (1:1, v/v) total extracts was mixed with 2.4 mL of working solution and stored in dark for 1 h at room temperature. The absorbance was determined at 593 nm. The results were expressed as mmol Fe^{2+} per 100 g fresh weight of samples.

2.6. Cell Viability

The Caco-2 human epithelial cell line (ATCC HTB-37) was cultured with high-glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cell viability was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described with slight modification [23]. Caco-2 cells (1.0×10^4 cells/well) were seeded in 96-well plates and cultured for 24 h. Cells were treated with 80 µL total extracts (2.5 µg/mL) of wheat sprouts (Day 0) and microgreen (Day 6) for 4h firstly, and then 20 µL lipopolysaccharide (LPS, 1 µg/mL) was added to each well. After incubation for additional 24 h, cells were incubated with MTT (1 mg/ml) for 2 h at 37 °C. The medium was replaced with 100 µL of isopropanol and the absorbance was measured at 550 nm using a micro-plate reader.

2.7. Statistical Analysis

Statistical analyses were performed using a one-way analysis of variance (ANOVA) test and general linear models with the software SPSS, version 23.0 (SPSS Inc., Chicago, IL, USA). Results were expressed as the mean ± SD.

3. Results

3.1. Growth Parameters of Wheat Sprouts and Microgreens

Four wheat cultivars (Verna, Timilia, Sagittario and Claudio) were harvested at 0, 1, 4, 5, 6 days after germination in the dark for 48 h. Shoot length, fresh weight and dry matter percentage are important factors showing growth quality. These growth parameters of the wheat sprouts and microgreens grown under the LED and FL light were reported in Figure 1 and Table 1.

Sprouts are obtained from the seeds germinated for 48 h under the dark (Day 0). Significant differences were showed in growth parameters among the four cultivars. Claudio and Verna showed both higher shoot length and fresh weight than Timilia and Sagittario. While the dry matter percentage of Claudio and Sagittario were significantly different from Timilia and Verna. After exposure under two different lights for 1 day, the shoot length and fresh weight of wheat sprouts were not significantly affected by two different lights. While higher dry matter percentage was showed in Sagittario and Claudio sprouts grown under LED light compared to FL light (Supplementary Table 1).

According with the definition of microgreens, the last group were harvested at Day 6, within a pair of true leaves. The shoot length, fresh weight and dry matter percentage of wheat microgreens obtained from the last harvest day ranged between 142.10 – 203.75 mm, 2.48 – 3.80 g and 9.34 – 12.48%, respectively. Microgreens developed under FL light from all cultivars were shown longer shoot length than those under LED light. Verna grown under FL showed the highest shoot length among all the cultivars selected. However, there is no significant difference showed in the fresh weight of the four cultivars between the two light conditions. The fresh weight of Timilia was higher than the other three cultivars. The dry matter percentage was significantly affected by the two different light treatments in all microgreens, the higher dry matter percentage were showed in microgreens grown under LED light in comparison with FL light.

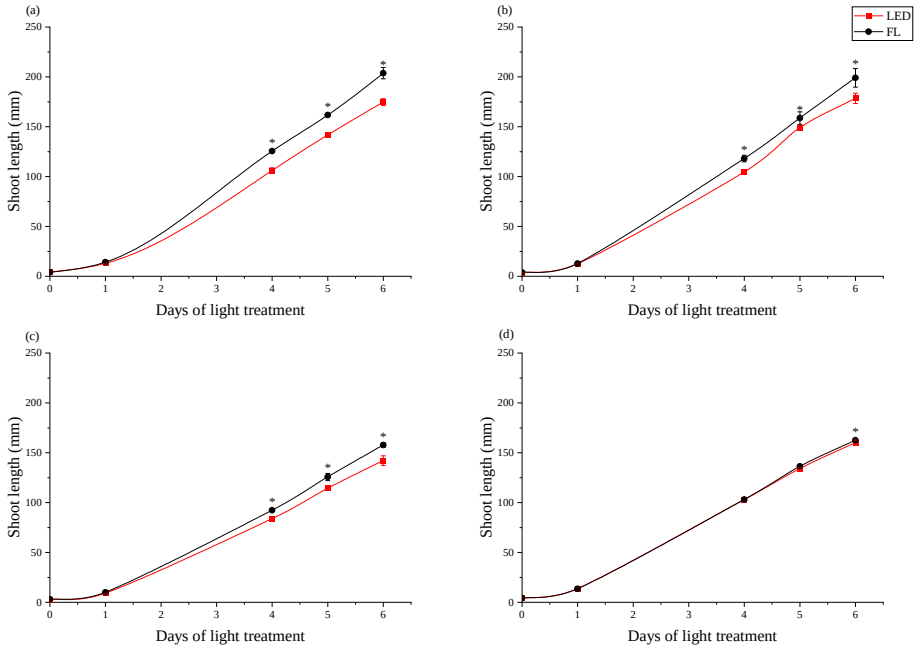

Fig. 1. The shoot length of sprouts and microgreens obtained from (a) Verna, (b) Timilia, (c) Sagittario, (d) Claudio grown under LED and FL light. Red lines indicate microgreens grown under LED light and black lines indicate microgreens grown under FL light. The error bars indicate the standard deviation. The "*" represents significant difference between two different light treatments ($p < 0.05$).

Table 1. Growth parameters of wheat sprouts and microgreens from four cultivars (Verna, Timilia, Sagittario and Claudio) at 0 and 6 days. Standard errors in the brackets. The different lowercase letters in each column denote significant differences among cultivars for sprouts and microgreen respectively at $p < 0.05$.

Variety		**Shoot length (mm)**	**Fresh weight (g)**	**Dry matter (%)**
Sprouts				
Verna		4.11 (±0.29)a	0.73 (±0.00)a	54.57 (±0.99)c
Timilia		3.62 (±0.29)b	0.63 (±0.07)b	58.30 (±0.36)b
Sagittario		2.94 (±0.16)c	0.61 (±0.04)b	62.84 (±3.05)a
Claudio		4.11 (±0.40)a	0.78 (±0.04)a	64.87 (±1.43)a
Microgreens				
Verna	LED	174.70 (±3.45)b	2.75 (±0.39)cd	12.48 (±0.70)a
	FL	203.75 (±5.66)a	2.99 (±0.09)bcd	10.56 (±0.49)bc
Timilia	LED	178.50 (±5.15)b	3.80 (±0.20)a	10.84 (±0.57)b
	FL	199.05 (±9.35)a	3.73 (±0.48)a	9.34 (±0.20)e
Sagittario	LED	142.10 (±4.82)d	2.62 (±0.49)cd	11.14 (±0.38)b
	FL	157.65 (±2.25)c	2.48 (±0.15)d	9.52 (±0.05)de
Claudio	LED	160.10 (±2.81)c	3.57 (±0.27)ab	10.93 (±0.19)b
	FL	162.50 (±1.35)c	3.09 (±0.17)bc	10.07 (±0.20)cd
Significance	G	***	***	***

L	***	ns	***
G×L	***	ns	ns

(* indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$; *** indicates significance at $p < 0.005$; ns = not significant)

3.2. Shoot Pigments of Wheat Sprouts and Microgreens

The shoot pigments were analyzed with total chlorophyll (chlorophyll a + chlorophyll b) and 5×total carotenoid, which was showed in Figure 2. The detailed data set of pigments (chlorophyll a, chlorophyll b and carotenoid) from wheat sprouts and microgreens at two different lights were available in Table 2. Significant increase of pigment contents was observed along the successive growth days of wheat sprouts and microgreens obtained from the four cultivars in the two different light conditions. However, the rate of accumulation decreased at day 4 or 5 for all of the wheat microgreens of four cultivars selected.

There was significant difference of pigment contents shown among the four cultivars of wheat sprouts. In particular, Verna and Sagittario sprouts showed a higher content of chlorophyll a, chlorophyll b and carotenoids than Timilia and Claudio. The highest pigment content was showed in Verna and reached the value of 40.22 μg/g FW. After exposure to lights for 1 day, chlorophyll contents of wheat sprouts were not significantly affected by two different lights. However, the content of carotenoids ascertained in Verna, Sagittario and Claudio grown under LED light were higher than those under FL light (Supplementary Table 2).

Wheat microgreens grown under LED light showed higher chlorophyll a and carotenoid content than those under FL light at Day 6. However, the chlorophyll b content was less affected by the two different lights. The highest pigment content was showed in Verna grown under LED light and reached the value of 1464.95 μg/g FW with both the highest content of chlorophyll a (890.86 μg/g FW) and carotenoids (216.95 μg/g FW). The lowest pigment content was showed in Timilia grown under FL light and reached the value of 1000.88 μg/g FW.

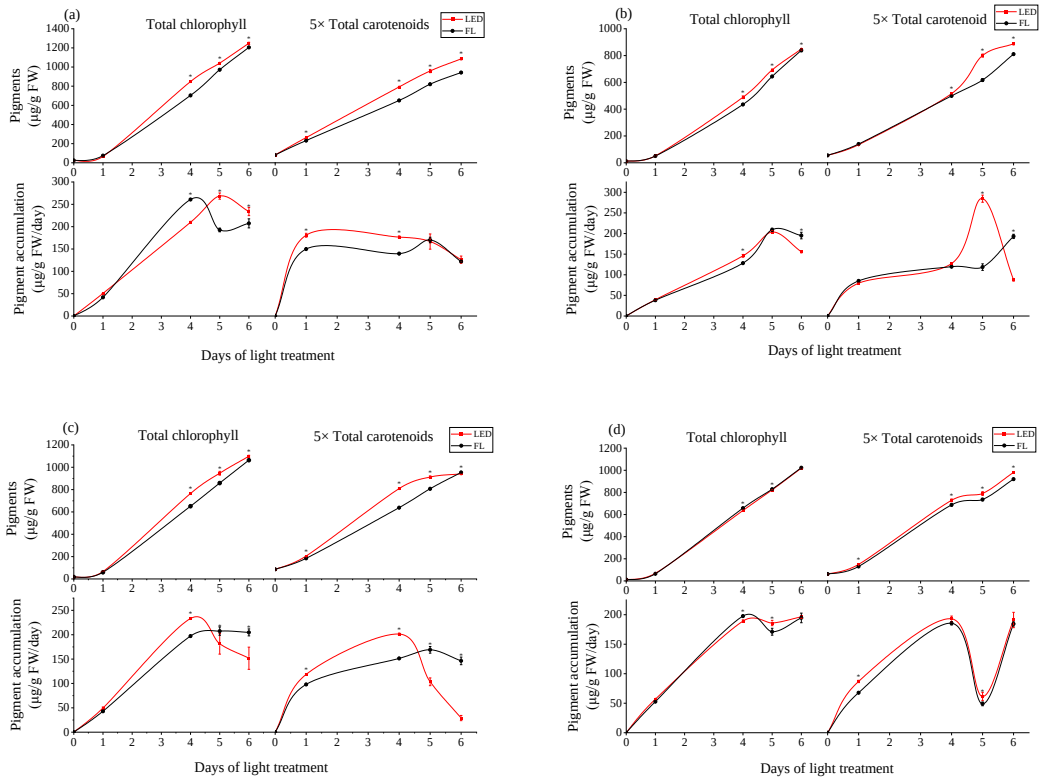

Fig. 2. Chlorophyll and 5 × Carotenoid contents and their rate of accumulation in (a) Verna, (b) Timilia, (c) Sagittario, (d) Claudio sprouts and microgreens with days of light treatment. The error bars indicate the standard deviation. The "*" represents significant difference between LED and FL light treatments ($p < 0.05$).

Table 2. Pigment contents of wheat sprouts and microgreens from four cultivars (Verna, Timilia, Sagittario and Claudio) at 0 and 6 days. Standard errors in the brackets. The different lowercase letters in each column denote significant differences among cultivars for sprouts and microgreen respectively at $p < 0.05$.

Variety		Chlorophyll a (µg/g FW)	Chlorophyll b (µg/g FW)	Carotenoid (µg/g FW)
Sprouts				
Verna		11.85 (±0.25)a	12.07 (±0.32)a	16.30 (±0.12)b
Timilia		5.22 (±0.44)c	6.59 (±0.85)c	10.96 (±0.03)d
Sagittario		8.57 (±0.62)b	8.07 (±1.20)b	17.25 (±0.28)a
Claudio		5.95 (±0.17)c	5.69 (±0.43)c	12.47 (±0.06)c
Microgreens				
Verna	LED	890.86 (±6.81)a	357.14 (±4.05)b	216.95 (±1.76)a
	FL	840.64 (±4.01)b	364.88 (±3.22)a	188.48 (±1.73)cd
Timilia	LED	616.87 (±2.60)f	231.27 (±2.89)e	177.54 (±1.59)f
	FL	606.72 (±4.66)g	232.08 (±5.06)e	162.08 (±1.03)g
Sagittario	LED	784.40 (±4.20)c	313.91 (±4.36)c	188.15 (±1.23)d
	FL	754.30 (±2.36)d	308.44 (±3.75)c	190.68 (±0.31)c
Claudio	LED	739.21 (±0.84)e	279.07 (±1.38)d	196.15 (±0.81)b

	FL	738.93 (±2.38)e	284.00 (±2.27)d	183.96 (±1.40)e
Significance	G	***	***	***
	L	***	ns	***
	G×L	***	*	***

(* indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$; *** indicates significance at $p < 0.005$; ns = not significant)

3.3. Polyphenol Contents of Wheat Sprout and Microgreen Extracts

The TPC and TFC of wheat sprouts and microgreens from Verna, Timilia, Sagittario and Claudio grown under two different light conditions were presented in Table 3. As mentioned above, wheat sprouts including shoot, seed coat and roots were taken after 48 h germination under the dark (Day 0) and exposure to light for 1 day (Day 1), while microgreens were taken from 1 cm above the pot, only the shoot, for all the analysis of total aqueous extracts.

Significant differences of polyphenol contents were observed among the four cultivars of wheat sprouts. The highest TPC was shown in Verna with the value of 104.79 mg/100 g FW, followed by Claudio and Sagittario. While Timilia showed a higher level of TFC than other three cultivars and reached the value of 17.14 mg/100 g. After exposure to lights for 1 day, there was a sharp increase for the TPC and TFC of wheat sprouts from Day 0 to Day 1. In general, the TPC and TFC of Claudio sprouts were significantly affected by two different lights (Supplementary Table 3).

Regarding microgreens, LED light could increase of TPC and TFC compared to FL light. The TPC of Verna, Timilia, Sagittario and Claudio microgreens grown under LED light at the last harvest day of light treatment were 63.81%, 35.50%, 49.69%, 39.58% respectively higher than those under FL light control. The TPC of Claudio grown under LED significantly differed from other three cultivars, with the highest value of 186.84 mg/100 g FW. Timilia and Claudio grown under LED light showed higher TFC value than Verna and Sagittario.

Table 3. Polyphenol contents and antioxidant activities of wheat sprouts and microgreens from four cultivars (Verna, Timilia, Sagittario and Claudio) at 0 and 6 days. Standard errors in the brackets. The different lowercase letters in each column denote significant differences among cultivars for sprouts and microgreen respectively at $p < 0.05$.

Variety		TPC (mg/100 g FW)	TFC (mg/100 g FW)	DPPH (μmol trolox/g FW)	FRAP (mg/100 g FW)
Sprouts					
Verna		104.79 (±4.59)a	14.73 (±0.32)b	1.92 (±0.02)a	0.63 (±0.03)a
Timilia		70.40 (±2.37)c	17.14 (±1.00)a	0.85 (±0.02)d	0.42 (±0.01)d
Sagittario		89.53 (±3.80)b	12.26 (±0.32)c	1.20 (±0.04)c	0.53 (±0.01)c
Claudio		90.23 (±2.13)b	14.08(±0.48)b	1.49 (±0.00)b	0.57 (±0.01)b
Microgreens					
Verna	LED	150.88 (±6.34)c	47.79 (±6.28)b	1.72 (±0.04)e	0.90 (±0.10)e
	FL	92.11 (±2.63)g	33.99 (±2.80)d	1.72 (±0.02)e	0.62 (±0.03)g
Timilia	LED	172.11 (±2.11)b	59.18 (±1.31)a	3.66 (±0.20)b	1.23 (±0.04)b
	FL	127.02 (±2.65)e	34.73 (±1.63)d	3.16 (±0.04)c	1.05 (±0.03)d
Sagittario	LED	168.07 (±4.48)b	42.88 (±0.98)c	3.27 (±0.00)c	1.09 (±0.02)d
	FL	112.28 (±2.60)f	28.81 (±1.69)e	2.75 (±0.06)d	0.80 (±0.04)f
Claudio	LED	186.84 (±1.82)a	51.31 (±2.93)b	4.44 (±0.25)a	1.42 (±0.02)a
	FL	133.86 (±0.61)d	38.25 (±0.48)cd	4.51 (±0.06)a	1.30 (±0.01)b
Significance	G	***	***	***	***
	L	***	***	***	***
	G×L	*	**	***	***

(* indicates significance at $p < 0.05$; ** indicates significance at $p < 0.01$; *** indicates significance at $p < 0.005$; ns = not significant)

3.4. Antioxidant Activity of Wheat Sprout and Microgreen Extracts

Antioxidant activity of wheat sprouts and microgreens grown under two different light conditions showed in DPPH and FRAP assay are presented in Table 3. In general, both assays were comparable. The antioxidant activity determined by the FRAP assay was similar to the DPPH assay, such as Claudio microgreens showed the highest value both in DPPH and FRAP assay among the four cultivars selected.

The antioxidant activity was significantly different among wheat sprouts from four cultivars. Verna and Claudio showed the higher antioxidant activity than Sagittario and Timilia both in DPPH and FRAP assay. The highest level of DPPH and FRAP was shown in Verna sprout with the value of 1.92 μmol trolox/g FW and 0.63 mg/100 g FW, respectively. After exposure to lights for 1 day, there was a significant increase for the DPPH and FRAP value of wheat sprouts, about 2-fold increase. However, the LED light

did not cause a statistically significant difference compared to FL light (Supplementary Table 4).

Regarding of DPPH assay, Timilia and Sagittario microgreens developed under LED light showed significantly higher values than those under FL light at the last harvest day. Claudio grown under FL light on the Day 6 showed the higher value (4.51 μmol trolox/g FW) than other cultivars, while there was no significant difference with that LED light (4.44 μmol trolox/g FW). Concerning to FRAP assay, the LED light caused a statistically significant difference compared to FL light for all wheat microgreens. Claudio grown under LED light showed the highest level among all the cultivars on the Day 6 and reached the value of 1.42 mg/100 g FW, followed by Timilia and Sagittario.

3.5. The Effect of Wheat Sprouts and Microgreens on Cell Viability of Caco-2 Cells

In the present study, we preferred to have a pretreatment with extracts firstly, and then induce inflammation (1 μg/mL LPS) to investigate the inflammatory preventive effect of wheat sprouts and microgreens on cell viability of Caco-2 cells. As intestinal epithelial cells, Caco-2 is a good alternative to animal studies for predicting intestinal absorption, which is very important for evaluation the nutritional potential of polyphenols and other bioactive compounds. The MTT assay outlined that cell survival was significantly reduced by the presence of LPS compared to control group (Figure 3). The bioprotective effects against LPS-induced damage to Caco-2 cells were observed in the treatment with all the microgreens extracts. Cell survival significantly increased by the treatment of Sagittario and Claudio microgreens, with an average of 107% and 111% of the control, respectively. Sagittario microgreens grown under LED light showed the best bioprotective effective among four cultivars and reached the value of 112% of the control, also significantly different from those under FL light. However, cell survival was not significantly influenced by the treatment of wheat sprouts.

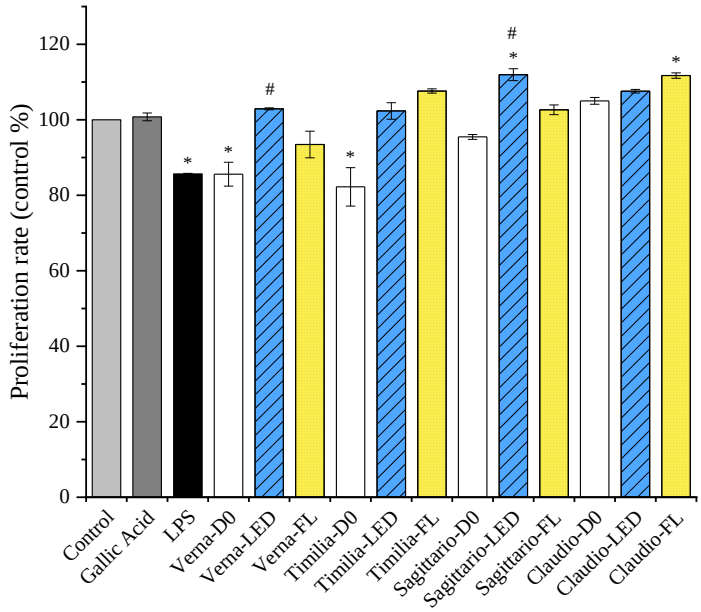

Fig. 3. Effect of total aqueous extracts from wheat sprouts (D0 : Day 0) and microgreens (Day 6) on cell viability of Caco-2 cells. The error bars indicate the standard deviation. (The "*" represents significant difference compared to control at $p < 0.05$. The "#" indicates significance between LED and FL light at $p < 0.05$.)

4. Discussion

In the present study, we investigated the growth parameter, pigment contents, polyphenol contents, antioxidant activities and the effects on cell viability of wheat sprouts and microgreens from four cultivars, Verna, Timilia, Sagittario, and Claudio. Different light condition can partially influence plants growth, the contents of photosynthetic pigments and antioxidants in some specific species. However, there was no specific trend in term of ancient and modern, or soft and durum wheat sprouts and microgreens.

4.1. Growth Parameters of Wheat Spouts and Microgreens

There was no significant effect of light on shoot length of sprouts may because of the short time of light treatment (only 1 day). This confirms evidences from the research which reported a lack of significant effect of light on wheat sprouts of einkorn and emmer [3]. LED light also failed to increase the shoot length of all wheat microgreens. This observation may be associated with the differences of spectral composition such as different ratio of red and blue light. A negative linear correlation was found between

blue light and height of wheatgrass [17]. It was reported that buckwheat shoots grown under FL light were longer than those under blue LED light but shorter than those under red LED light after exposure to light for 5 days [24].

Fresh and dry weight are also important factors showing growth quality. The effects of light on fresh and dry weight varies depend upon the light spectra and sprouts and microgreen species. It was reported that fresh shoot weight of wheatgrass increased by decreasing the fraction of blue LED light [17]. LED UV-A light was shown to decrease the fresh weight of beet microgreens, while increase the fresh weight of basil microgreens [25]. However, there was no significant difference of LED and FL light on fresh weight was observed in our study. However, it was shown a significant effect of light on dry matter percentage for four wheat cultivars.

4.2. Shoot Pigments of Wheat Sprouts and Microgreens

Differences in the light spectra can influence pigment contents because various species of plant possess different ability to perceive the differences in light quality through their photoreceptors [17]. It is well known that chlorophylls show large absorption in the red and blue regions [26]. Generally, LED light is beneficial for the increase of pigments in wheat sprouts and microgreens. Our experiment indicated a significant effect of the LED light on the content of chlorophyll and carotenoids in wheat microgreens at the last harvest day compared to FL light, it was possibly explained that the LED light used in our study showed a wider emission range in red and blue compared to FL light. Similarly, the chlorophyll content of einkorn wheat was stimulated effectively with a combination of red and blue LED light [27]. Carotenoids act as accessory light-harvesting pigments, which also play essential roles in photosynthetic organisms by transmitting energy to chlorophylls. It was reported that the supplemental blue, red and green LED light could increase the content of α-carotene and β-carotene of microgreens [4].

A significant increase of pigment contents ($p < 0.05$) was observed in the initial growth days under two light conditions for four wheat cultivars, which could indicate the photosynthetic and metabolic activity of wheat sprouts and microgreens [28]. The total Chlorophyll and carotenoid contents were not arrived the peak in the study period

in the present research, but the rates of accumulation were arrived or started to fall with an upcoming peak. The highest value of pigment accumulation indicated the maximum activities of biosynthetic enzymes and energy requirement by the cell. A possible reason for the decreased rate of accumulation of pigment after the peak is that phenolic and other antioxidants produced rapidly in wheat sprout and microgreens during this period. The biosynthesis of pigments is decreased by increased amount of allelopathic phenolics [14].

4.3. Total Polyphenol and Antioxidant Activity of Aqueous Extracts

Our results confirmed the four cultivars of wheat sprouts and microgreens contained large amount of phenolic and flavonoid contents of wheat sprouts and microgreens, subsequently showed a significant scavenging power in both FRAP and DPPH radical assay. Similar results were reported by Parit [29], which demonstrated that wheatgrass extracts obtained after germination for 8 days showed significant antioxidant activity both in DPPH and FRAR assay. Significant differences of polyphenol contents and antioxidant activities of microgreens were showed among four cultivars in our study. Similarly, Chen found that phenolic content, profile and antioxidant activity of sprouted wheat significantly differed from various Chinese wheat cultivars and gemination stage [30].

Previous studies demonstrated that light could improve photosynthesis and the malonyl-CoA pathway, subsequently increases synthesis of phenolic compounds in sprouts. Moreover different light has different effects on polyphenol contents [31]. In general, our results showed the beneficial role of LED light in the improvement of polyphenol contents and antioxidant activities of wheat sprouts and microgreens compared to FL light, which was consistent with previous report [32]. The effect of LED light spectral qualities on modulation of secondary metabolites and their synthesis in microgreens were reported by many studies [33]. However, studies on the effect of different light on polyphenol contents and antioxidant activities are limited for sprouted wheat. The H_2O_2 and MDA content of einkorn wheatgrass was reduced effectively with a combination of red and blue LED light with an appropriate proportion of blue light

[17]. It was also reported that supplementation with green light promoted antioxidant activities of wheat-sprouted seeds [15].

4.4. The Effect of Wheat Sprouts and Microgreens on Cell Viability of Caco-2 Cells

It was well demonstrated many benefits of wheatgrass from previous studies. In particular, several studies have described the anti-inflammation effect of wheatgrass. It was reported that wheatgrass juice has significant anti-inflammation activity against rexin pellet-induced granuloma in albino rats [34]. LPS would stimulate cells to release pro-inflammatory cytokines, like interleukin (IL)-1 and IL-6, tumor necrosis factor (TNF)-α, which would cause an injury of organ eventually. Polysaccharide obtained from wheatgrass showed a protective effect on hepatic cell death introduced by LPS, inhibited up-regulation of pro-inflammatory cytokine and improved the oxidative status of liver [35]. Wheatgrass shoots were also reported that could increase cell viability of Caco-2 cells from H_2O_2-induced damage in MTT assay [36]. Similarly, our results of MTT assay also suggested that wheat microgreens from four cultivars prevent the damage caused by LPS to Caco-2 cells, which confirmed their preventive inflammatory potentials. Some cultivars showed significant protective effects on cell survival. It was possibly explained by the difference of polyphenol and pigment contents, or other antioxidants contained in various wheat microgreens grown on different light.

5. Conclusion

The present study is the first study that addressed the difference of LED and FL light that we used to produce wheat sprouts and microgreens in a controlled environment. The effect of light was generally not significant for wheat sprouts, while different light condition can partially influence growth, the contents of photosynthetic pigments and antioxidants in some specific species of wheat microgreens. Overall, the results obtained in this study suggested that LED light failed to increase the shoot length and fresh weight, however, increase the dry matter percentage of wheat microgreens, when compared to FL light. LED light significantly increased the Chlorophyll a, carotenoid, total phenolic and total flavonoid contents, as well as improved the antioxidant activities shown in the FRAP assay and bioprotective potential on Caco-2 cells of wheat

microgreens, when compared to FL light. LED light is optimal culture condition to produce wheat microgreens. Among the four cultivars of wheat microgreens grown under LED light, Verna showed the highest pigment content, while Claudio exhibited the highest TPC and antioxidant properties. However, further research, especially the different proportion of red and blue LED light, as well as more evidence from bioprotective activity of wheat microgreens that are cultivated using LED light, are required to choose the ideal growth condition to develop wheat microgreens.

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Supplementary Table 1

Variety	Days	Shoot length (mm)		Fresh weight (g)		Dry matter (%)	
		LED	FL	LED	FL	LED	FL
Verna	0	4.11±0.29	4.11±0.29	0.73±0.00	0.73±0.00	54.57±0.99%	54.57±0.99%
	1	12.99±0.62	14.11±0.31	0.80±0.05	0.75±0.02	46.68±0.77%	47.74±0.45%
	4	106.10±2.72	125.48±0.90	1.46±0.05	1.68±0.05	10.97±0.19%	9.88±0.13%
	5	141.88±2.37	161.70±0.85	2.12±0.48	2.43±0.24	11.60±0.77%	9.55±0.21%
	6	174.70±3.45	203.75±5.66	2.75±0.39	2.99±0.09	12.48±0.70%	10.56±0.49%
Timilia	0	3.62±0.30	3.62±0.30	0.63±0.07	0.63±0.07	58.30±0.36%	58.30±0.36%
	1	12.44±0.50	12.78±0.53	0.68±0.06	0.68±0.07	51.84±1.47%	52.59±0.39%
	4	104.45±2.08	118.07±3.33	1.41±0.16	2.14±0.15	10.53±0.77%	8.70±0.25%
	5	149.08±0.60	158.63±6.26	2.73±0.21	2.82±0.22	10.24±0.18%	9.17±0.26%
	6	178.50±5.15	199.05±9.35	3.80±0.20	3.73±0.48	10.84±0.57%	9.34±0.20%
Sagittario	0	2.94±0.16	2.94±0.16	0.61±0.04	0.61±0.04	62.84±3.05%	62.84±3.05%
	1	8.95±0.39	9.94±0.46	0.69±0.01	0.73±0.02	51.50±1.41%	48.30±0.28%
	4	83.77±0.19	92.22±0.66	1.21±0.08	1.55±0.24	10.27±0.30%	9.40±0.90%
	5	114.28±0.11	125.68±3.57	1.67±0.07	2.07±0.26	10.57±0.27%	9.19±0.31%
	6	142.10±4.82	157.65±2.25	2.62±0.49	2.48±0.15	11.14±0.38%	9.52±0.05%
Claudio	0	4.11±0.40	4.11±0.40	0.78±0.04	0.78±0.04	64.87±1.43%	64.87±1.43%
	1	13.69±0.36	13.55±0.36	0.96±0.03	0.98±0.06	50.04±1.26%	47.88±1.34%
	4	102.90±2.19	103.02±2.03	1.76±0.17	1.78±0.12	10.08±0.34%	9.57±0.14%
	5	134.08±1.11	136.30±0.28	2.91±0.20	2.61±0.11	10.28±0.41%	9.72±0.18%
	6	160.10±2.81	162.50±1.35	3.57±0.27	3.09±0.17	10.93±0.19%	10.07±0.20%

Supplementary Table 2

Variety	Days	Chlorophyll a		Chlorophyll b		Carotenoids	
		LED	FL	LED	FL	LED	FL
Verna	0	11.85±0.25	11.85±0.25	12.07±0.32	12.07±0.32	16.30±0.12	16.30±0.12
	1	52.72±1.02	58.43±0.11	12.90±0.17	15.94±0.18	52.46±0.93	46.29±0.28
	4	608.02±2.07	498.03±3.74	239.88±4.43	205.21±2.83	158.37±0.96	130.08±0.26
	5	732.05±2.28	683.16±2.01	308.54±1.72	288.50±2.09	191.70±3.33	164.11±1.41
	6	890.86±6.81	840.64±4.01	357.14±4.05	364.88±3.22	216.95±1.76	188.48±1.73
Timilia	0	5.22±0.44	5.22±0.44	6.59±0.85	6.59±0.85	10.96±0.03	10.96±0.03
	1	31.62±0.44	32.89±0.22	19.64±0.82	17.26±0.53	26.83±0.04	27.95±0.09
	4	341.35±4.25	313.53±1.79	147.54±5.89	121.14±2.14	103.09±0.72	99.72±0.59

	5	497.33±3.45	452.10±1.45	195.14±3.98	191.58±1.50	159.99±2.17	123.46±2.03
	6	616.87±2.60	606.72±4.66	231.27±2.89	232.08±5.06	177.54±1.59	162.08±1.03
Sagittario	0	8.57±0.62	8.57±0.62	8.07±1.20	8.07±1.20	17.25±0.28	17.25±0.28
	1	50.64±0.17	45.82±0.27	15.40±0.46	13.71±0.43	40.93±0.06	36.82±0.10
	4	549.12±2.73	464.54±2.60	216.05±2.28	186.15±2.33	161.68±1.16	127.62±0.51
	5	680.29±7.08	608.74±2.93	266.25±9.99	249.28±4.97	182.36±2.19	161.42±1.56
	6	784.40±4.20	754.30±2.36	313.91±4.36	308.44±3.75	188.15±1.23	190.68±0.31
Claudio	0	5.95±0.17	5.95±0.17	5.69±0.43	5.69±0.43	12.47±0.06	12.47±0.06
	1	35.51±0.27	35.51±0.54	33.01±0.28	28.82±0.86	29.85±0.42	26.02±0.10
	4	455.20±2.28	464.73±2.05	180.84±4.15	192.48±0.98	145.65±2.47	137.31±1.66
	5	584.07±2.25	582.41±4.61	226.29±0.95	245.96±2.92	150.23±3.22	147.09±2.12
	6	739.21±0.84	738.93±2.38	279.07±1.38	284.00±2.27	196.15±0.81	183.96±1.45

Supplementary Table 3

Varieties	**Days**	**TPC (mg/100 g)**		**TFC (mg/100 g)**	
		LED	**FL**	**LED**	**FL**
Verna	0	104.79±4.59	104.79±4.59	14.73±0.32	14.73±0.32
	1	254.04±1.61	259.65±5.68	35.78±0.17	32.34±1.22
	4	102.68±4.59	72.86±1.69	12.26±0.32	9.46±0.49
	5	107.77±3.08	87.77±0.61	43.25±1.95	39.52±0.80
	6	150.88±6.34	92.11±2.63	47.79±6.28	33.99±2.80
Timilia	0	70.40±2.37	70.40±2.37	17.14±1.00	17.14±1.00
	1	190.00±3.97	197.72±2.13	19.62±0.17	19.92±0.17
	4	118.12±4.89	97.95±2.29	15.05±0.49	11.83±0.49
	5	151.81±3.99	114.44±0.80	56.59±1.83	59.72±1.83
	6	172.11±2.11	127.02±2.65	59.18±1.31	34.73±1.63
Sagittario	0	89.53±3.80	89.53±3.80	12.32±0.85	12.32±0.85
	1	205.96±1.22	207.19±2.90	21.43±0.93	22.65±2.80
	4	104.44±0.80	92.72±4.72	15.48±0.65	13.44±1.04
	5	122.68±2.79	124.30±1.99	31.64±3.87	35.88±1.39
	6	168.07±4.48	112.28±2.60	42.88±0.98	28.81±1.69
Claudio	0	90.23±2.13	90.23±2.13	14.08±0.48	14.08±0.48
	1	219.12±3.73	255.09±2.13	22.75±0.35	28.71±1.15
	4	142.16±3.45	151.49±0.80	26.67±1.04	29.78±1.78
	5	166.89±4.18	157.46±3.17	52.34±0.70	38.81±2.61
	6	186.84±1.82	133.86±0.61	51.31±2.93	38.25±0.48

Supplementary Table 4

Varieties	**Days**	**DPPH (µmol trolox/g)**		**FRAP (mg/100 g)**	
		LED	**FL**	**LED**	**FL**
Verna	0	1.92±0.02	1.92±0.02	0.63±0.03	0.63±0.03
	1	2.84±0.04	4.05±0.06	1.55±0.33	1.59±0.01

	4	1.98±0.08	1.44±0.02	0.92±0.02	0.71±0.01
	5	1.23±0.06	1.69±0.17	0.90±0.05	0.77±0.11
	6	1.72±0.04	1.72±0.02	0.90±0.10	0.62±0.03
Timilia	0	0.85±0.02	0.85±0.02	0.42±0.01	0.42±0.01
	1	2.31±0.02	2.76±0.02	1.14±0.01	1.25±0.01
	4	2.73±0.04	2.29±0.06	1.00±0.05	0.89±0.01
	5	3.91±0.08	1.69±0.17	1.17±0.03	0.93±0.01
	6	3.66±0.20	3.16±0.04	1.23±0.04	1.05±0.03
Sagittario	0	1.20±0.04	1.20±0.04	0.53±0.01	0.53±0.01
	1	2.82±0.06	3.06±0.13	1.34±0.21	1.31±0.07
	4	2.02±0.06	1.95±0.06	0.93±0.01	0.85±0.01
	5	2.76±0.10	2.96±0.08	1.00±0.02	0.88±0.01
	6	3.27±0.00	2.75±0.06	1.09±0.02	0.80±0.04
Claudio	0	1.49±0.00	1.49±0.00	0.57±0.01	0.57±0.01
	1	4.96±0.08	4.88±0.04	1.47±0.09	2.03±0.03
	4	3.46±0.04	4.48±0.04	1.39±0.03	1.54±0.02
	5	4.27±0.06	4.77±0.23	1.45±0.01	1.40±0.03
	6	4.44±0.25	4.51±0.06	1.42±0.02	1.30±0.01

Chapter 4 - Are Supplements Safe? Effects of Gallic and Ferulic Acids on In Vitro Cell Models

Abstract

Polyphenols display health-promoting properties linked to their biological activities. They are initially absorbed in the small intestine, then they are largely metabolized in the colon, whereupon they are able to exert systemic effects. The health-promoting properties of polyphenols have led to the development of food supplements, which are also largely consumed by healthy people, even if data on their safety are still yet lacking. In the present paper, the content of gallic acid and ferulic acid was analyzed in two supplements, and shown to be higher than the relative contents found in fruit and flour. To evaluate the effects of these phenolic compounds on epithelial intestinal tissue, gallic and ferulic acids were added to a new in vitro model of the intestinal wall at different concentrations. The effects on viability, proliferation and migration of these compounds were respectively tested on three different cell lines (Caco2, L929 and U937), as well as on a tridimensional intestinal model, composed of a mucosal layer and a submucosa with fibroblasts and monocytes. Results indicated that gallic and ferulic acids can exert toxic effects on in vitro cell models at high concentrations, suggesting that an excessive and uncontrolled consumption of polyphenols may induce negative effects on the intestinal wall.

Keywords: supplements; gallic acid; ferulic acid; intestinal wall models

1. Introduction

Polyphenols are natural compounds, chemically characterized by phenolic structural units. They are known to have health properties linked to their biological activities [1]. These compounds are mainly produced by plants as secondary metabolites, and act as antioxidants by reacting with reactive oxygen species (ROS). Moreover, polyphenols may be involved in gene regulation on a cellular level [2]. Polyphenols are divided into two principal subgroups: flavonoids (flavones, isoflavones, flavonols, flavanones, flavan-3-ols, anthocyanins and condensed tannins), with a primary structure consisting of two benzene rings connected by a pyrone C-ring, and non-flavonoids (benzoic acids, stilbenes, lignans, gallotannins, hydrolysable tannins and ellagitannins), with more heterogeneous and complex structures [3]. Phenolic compounds are not readily available, since only a small percentage is digested and adsorbed in the small intestine. They are predominantly metabolized by gut microbiota in the colon. Resulting polyphenol metabolites are then adsorbed, after which they are known to exert a systemic effect [4]. Polyphenols have been shown to perform free radical scavenging activities, related to their specific structural features, such as the number and position of hydroxyl and catechol units [5]. Due to these structural properties, polyphenols are known to reduce the prevalence of several oxidative stress markers, and thereby result in their beneficial roles in some pathological conditions, such as cardiovascular disease and diabetes [6,7]. An effect on the large bowel has also clearly been demonstrated, since polyphenols are able to promote healthy microbiota composition [8]. Microbiota modulation, in particular, has been linked to the ability of phenolic compounds to counteract colitis, induced by Dextran sodium sulphate, in mice [9]. A direct effect on intestinal cells has also been demonstrated. Caffeic acid and chlorogenic acid were reported to suppress TNF-α and IL-8 secretion in Caco2 cells [10]. Among the activities related to polyphenols, pro-oxidant activities [11] have also been reported. For instance, the spontaneous oxidation of gallic acid has been shown to generate several highly reactive species, including O_2^-, H_2O_2, quinones and semiquinones [12], and the generation of these species was dependent on both the polyphenol concentration and

the environment [13,14]. However, phenolic compounds have been primarily considered for their health-promoting properties. As a result, this has led to the development of an increasing number of food supplements, which are also largely ingested by healthy people to prevent non-communicable disease development and, more generally, to promote well-being. To date, however, no regulatory legislation exists to regulate polyphenol contents in food supplements, and data on the safety of these compounds are still lacking.

Although in vitro models are not optimal for evaluating the systemic effects of phenolic compounds, since they are largely metabolized by intestinal microflora and then adsorbed, a direct effect on the intestinal epithelium before their metabolization cannot be excluded. In order to evaluate the interaction between phenolic compounds and epithelial intestinal tissue, we developed a tridimensional in vitro model of colon tissue, and tested the effects of two of the non-flavonoid compounds, namely gallic acid and ferulic acid, on mucosal integrity, as well as their interaction with submucosal cellular components. The effects of these compounds were also tested on three different cell lines (Caco2, L929 and U937) to assess their effects on cell viability, toxicity, migration and apoptosis. Results indicated that gallic and ferulic acids can exert differing effects on cell models, according to their concentration and the cell type analyzed. However, they showed toxic effects in in vitro cell models at high concentrations, suggesting that an excessive and uncontrolled consumption of polyphenols could induce negative effects on intestinal mucosa.

2. Materials and Methods

2.1. Chemicals

3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio (MTT) assay was from Life Technologies (Carlsbad, CA, USA). Gallic acid, with purity of ≤ 100%, was from Sigma-Aldrich (Milan, Italy) and ferulic acid, with purity of ≥ 99%, was from Extrasynthese (Genay, France). Reagents for cell cultures, such as Hank's Balanced Salt Solution (HBSS), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), L-Glutamine, Penicillin-Streptomycin, RPMI-1640 Medium and rat tail

collagen type I were purchased from GIBCO (Waltham, MA, USA). Methanol and water (HPLC-grade, Lichrosolv®) were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade. The food supplements were based on the presence of a single raw material in addition to some excipients. The raw material of the first one was a dry extract obtained from an apple cultivar (Annurca), the second one was based on dry blueberry extract.

2.2. HPLC-MS/MS Analysis

Sample preparation: Standard stock solutions of gallic acid and ferulic acid, both at a concentration of 1 mg/mL, were prepared in water/methanol mixture (50/50, v/v). Working standard solutions were made by diluting stock solutions in water. Stock working solutions of the standards were stored at -20 °C. The extraction method used was as follows: 2 mL of methanol/acetone/water mixture (60/10/30, v/v/v) was added to 0.5 g of sample (dietary supplement). The mixture was stirred for 30 min and then centrifuged at 10,000 rpm for 10 min at 15 °C. The supernatant was filtered through a 0.22 μm nylon filter Before injection into the HPLC-MS (High Performance Liquid Chromatography), a blueberry-based dietary supplement and dietary supplement A were diluted (1:5 and 1:1, respectively) in water. Sample analysis: Samples were injected into a Waters e2695 Alliance HPLC System, coupled with a Waters ACQUITY QDa Mass Detector, as described by [15,16]. A Supelco_Analytical (Sigma-Aldrich St Louis, MO, USA) C18 15 cm × 4.6 mm, 5 μm was selected. The mobile phase consisted of (A) water + 0.1% acetic acid and (B) methanol. A gradient experiment was performed from 5% to 10% B in 4.17 min, from 10% to 20% B in 4.16 min and from 20% to 95% B in 14.67 min, respectively. Solvent B returned to 5% in 4 min and was maintained for 8 min to re-equilibrate the column. The flow rate, the injection volume and column temperature were 0.86 mL/min, 23.5 μL and 40 °C, respectively. The ionization source was used in the Electrospray Ionization ESI negative mode, using single ion recording (SIR). Optimal cone voltage was set at 15 V and the capillary voltage at 0.8 kV. The monitored (M–H)-ions were m/z 168.88 for gallic acid, and m/z 192.94 for ferulic acid. Data acquisition, data handling and instrument control were performed using the Empower 3 software.

2.3. Cell Model Systems

L929 mouse fibroblasts (ATCC-CCL1) were cultured with DMEM, to which 10% fetal bovine serum, 1 mM L-glutamine and 1% penicillin-streptomycin were added. The Caco2 human epithelial cell line (ATCC HTB-37), obtained from Colorectal adenocarcinoma, was cultured with DMEM, supplemented with 10%, fetal bovine serum and 1% penicillin-streptomycin. U937, a pro-monocytic, human myeloid leukemia cell line (ATCC CRL-1593.2), was cultured in RPMI-1640 Medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Stock solution of gallic and ferulic acids were prepared at the concentration of 1 mg/L in ethanol 70% v/v. Cells were treated, 24 h after seeding, with the following concentrations of gallic and ferulic acids: 2.5, 5, 10, 20 and 40 mg gallic acid equivalents (GAE)/L, respectively. The latter were diluted in DMEM for 24 h, after which their effects were evaluated in terms of cytotoxicity. Ethanol (70% v/v) was used as the control, suspended in DMEM alone.

2.4. MTT Viability Assay

Cell lines were treated either with five concentrations of gallic and ferulic acids, respectively, for 24 h, or in 70% ethanol as the control (suspended in DMEM alone). Adherent cells, as well as the L929 and Caco2 cells, were plated onto 96-well tissue culture plates (10^5 cells/well) in complete medium. After treatments, proliferative cells were detected using the MTT assay, according to the ISO 10993-5 International Standard procedure (ISO 10993-5, 2009). The MTT substrate was prepared in DMEM, then added to cells in culture to attain a final concentration of 1 mg/mL, and then incubated for 2 h in the culture incubator at 37 °C with 5% CO_2. After incubation, the medium was removed by aspiration. Isopropanol (100 µL) was added to each well and formazan dye formation was evaluated by a multi-well scanning spectrophotometer at 540 nm. Suspension cells, of the U937 cell line, were plated at a concentration of 10^5 cells/well onto 96-well culture plates in complete medium. After treatments, the cells were incubated with 0.5% MTT solution in PBS for 4 h at 37 °C, and then dissolved with 100 µL isopropanol in 0.04 N HCl. The plate was read at 540 nm and results were expressed as percentage of viable cells with respect to untreated controls (70% ethanol).

The percentage of cell proliferation was calculated using the following formula: (absorbance value of treated sample/absorbance value of control) ×100 = % of cell viability.

2.5. Cell Viability

Cell viability was measured using the blue trypan assay. The L929, Caco2 and U937 cells were each plated onto a 24-well tissue culture plate (5×10^6 cells/well) in complete medium. After 24 h, treatments diluted in DMEM were added to cells for another 24 h. To detect viability, cells were then carefully resuspended in a 0.4% Trypan Blue (Gibco) solution and vital cells were counted using Countess®II FL (ThermoFisher Scientific, Waltham, MA, USA). The results were expressed as a viability percentage of the control.

2.6. Caspase3/7 Detection

The L929, Caco2 and U937 cells were each plated onto a 24-well plate (10^4 cells/well). Both treated and untreated cells were resuspended in CellEvent. Caspase-3/7 Green Detection Reagent (ThermoFisher Scientific) for 30 min in PBS with 20% of FBS, according to a data sheet protocol. Cells were resuspended and analyzed using the Countess II FL Automated Cell Counter (ThermoFisher Scientific). Each sample was analyzed in duplicate.

2.7. Wound-Healing Assay

A total of 2×10^5 L929 fibroblasts were plated onto 24-well tissue culture plate. At 24 h after seeding, cells were washed three times in HBSS and a line for each well was drawn along the cell monolayer with a sterile plastic tip. Plates were washed twice with HBSS to remove all detached cells and incubated in DMEM with 5 mg gallic acid equivalents (GAE)/L gallic or ferulic acids or 70% ethanol as the control. Cells were monitored at 4 and 24 h from stimulation and pictures of cells were taken. The results of each experiment were expressed as the mean of migrated cells from three different areas. The final results were expressed as the mean of three different experiments.

2.8. Monocyte Migration

For the monocyte migration experiment, 50×10^4 U937 in each well was placed on the top of a 0.4 μm filter of tissue culture inserts (Transwell, Costar, Cambridge, MA,

USA) in 12-well plates. On the bottom of each well, 2×10^5 Caco2 cells were either plated or not plated. After a period of 24 h, Caco2 cell medium was replaced with DMEM together with 20 mg/L of either gallic or ferulic acid. After 4 h, filter supports were removed, stained with thiazine for just 10 s and cell migration counted manually using a microscope (6 fields for each condition). The experiment was repeated 3 times.

2.9. Intestinal Equivalents

For intestinal equivalents, 0.5 mL of a cell free collagen solution (1.35 mg/mL rat tail collagen type I in DMEM with 10% FCS and 1% Pen/Strep) was added to tissue culture inserts (Transwell, Costar, Cambridge, MA, USA) in 12-well plates. This pre-coated layer was overlaid with 1 mL of L929 fibroblasts (10^5/mL) together with monocytes (3×10^4/mL) mixed with collagen type I. After 2 h of incubation at 37 °C, 2×10^5 of Caco2 cells were seeded onto dermal reconstructs, and incubated at 37 °C with Caco2 medium, added both on the upper and the lower part of the filter support. After 5 days, intestinal equivalents were either treated or not treated with 5 or 20 mg/L of either gallic or ferulic acid, or 70% ethanol for the control, respectively. The intestinal equivalents were then fixed with formalin for 2 h at room temperature, dehydrated and embedded in paraffin.

2.10. Immunohistochemical Analysis

Paraffin-embedded intestinal equivalents were rehydrated. Sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and thiazine staining (Bio-Optica®, Milan, Italy). Occludin tissue expression was detected using a mouse anti-occludin primary antibody (Novus). Immunohistochemistry was performed using Fast Red chromogen. Stainings were performed according to the UltraVision LP Detection System AP Polymer & Fast Red Chromogen assay (Thermo Fisher Scientific, Waltham, MA, USA). Negative controls were obtained by omitting the primary antibody. The expression intensity was quantitatively determined using ImageJ software (Wayne Rasband). Signal detection was performed on at least 3 different sections and the results were reported as a mean.

2.11. Statistical Analysis

All the statistical analyses were performed using the Statistica 7.1 software (2005, StatSoft, Tulsa, OK, USA). The Bartlett's test, which verifies the basic assumption of ANOVA as the homogeneity of variances, was statistically significant ($p > 0.05$). The Duncan test, which is adequate in the case of non-homogeneity of varieties, was employed as a post hoc test. Linear discriminant analysis (LDA) was performed on a standardized matrix of the MTT results, viability and cell type tests, and included a total of 5 variables, namely: MTT activities and viability results on L929, Caco2 and U937 treated with firstly gallic acid, 2.5 (G2.5), 5 (G5), 10 (G10), 20 (G20) and 40 (G40) mg/L, and secondly, with ferulic acid 2.5 (F2.5), 5 (F5), 10 (F10), 20 (F20) and 40 (F40) mg/L; migration analysis on L929 treated firstly with gallic acid, 2.5 (G2.5), 5 (G5), 10 (G10), 20 (G20) and 40 (G40) mg/L, and secondly with ferulic acid, 2.5 (F2.5), 5 (F5), 10 (F10), 20 (F20) and 40 (F40) mg/L. Linear discriminant analysis is a multivariate technique that allows the scoring of cases as a function of the first two roots (canonical discriminant functions). This technique was used to visualize similarities and differences among genotypes [17]. It is also a statistical method used to find a linear combination of features (discriminant functions) that characterizes or separates two or more classes of objects or events. Linear discriminant analysis (LDA) was performed using Statistica 7.1 software (2005, StatSoft, Tulsa, OK, USA). The supervised learning technique was applied to the standardized data matrix of responses (vitality, migration and respiration MTT test) of the cell lines (Caco2, L929, U937) as a function of different concentrations of gallic and ferulic acids. The cases (gallic and ferulic acid at the doses of 0, 2.5, 5, 10, 20 and 40 mg/L) were scored according to the first two roots (canonical discriminant functions).

3. Results

3.1. Gallic and Ferulic Acid Content in Commercially Available Dietary Supplements

The two polyphenols, gallic and ferulic acids, which are highly expressed in fruits and cereals, respectively, were quantified in two commercially available dietary supplements, referred to as "Blueberry supplement" and "Supplement A", the last one

obtained from apple polyphenols extract. The objective of this study has been to understand the quantity of polyphenols that can be ingested via common dietary supplements. In both dietary supplements analyzed, gallic acid was present in a lower concentration compared to ferulic acid (Table 1). In particular, Blueberry supplement showed a concentration of gallic and ferulic acids of 0.3252 mg/g and 1.3695 mg/g, respectively. Supplement A displayed a concentration of gallic and ferulic acids of 0.6354 mg/g and 1.8494 mg/g, respectively (Table 1, Figure S1).

Table 1. Gallic and ferulic acid concentration analysis in two commercially available dietary supplements: Blueberry supplement and Supplement A.

	GALLIC ACID (mg/g) ± s.d.	FERULIC ACID (mg/g) ± s.d.
Blueberry Supplement	0.3252 ± 0.030	1.3695 ± 0.140
Supplement A	0.6354 ± 0.035	1.8494 ± 0.180

3.2. Effects of Gallic and Ferulic Acids on Mouse Fibroblast Proliferation and Migration

In order to understand the effects of gallic and ferulic acids on the proliferation and wound-healing ability of cellular components within the intestinal dermis, their effects on the L929 fibroblast's cell line have been studied. The cells were treated with increasing doses of gallic acid and ferulic acid (2.5, 5, 10, 20 and 40 mg/L), respectively, diluted in DMEM. The two phenolic acids showed differing effects, depending on the dose. No significant effect, attributable to the two phenolic acids at any dosage, was detected on cellular proliferation compared to the control (Figure 1a,b). Regarding the viability tests, a significant toxic effect was elicited by both gallic and ferulic acids at 40 mg/L (Figure 1c,d). Caspases 3/7 was not activated, by either gallic acid or ferulic acid stimulation, at 20 mg/L, compared to the untreated control (Figure 1e). The wound-healing assay showed that different concentrations of the two phenolic acids could either favor or not favor the shelter of intestinal tissue lesions. Cell migration was monitored over a period of 48 h, starting 4 h after addition of the stimulus, and terminating 48 h later. The most interesting results were observed 24 h after the treatments (Figure 2c). Ferulic acid showed a significant stimulating effect at 5, 10 and

20 mg/L, compared to the control, whereas a dose of 40 mg/L inhibited wound-healing (Figure 2a). Regarding gallic acid, 2.5 and 5 mg/L induced fibroblast migration, whereas this effect was inhibited at 40 mg/L (Figure 2b).

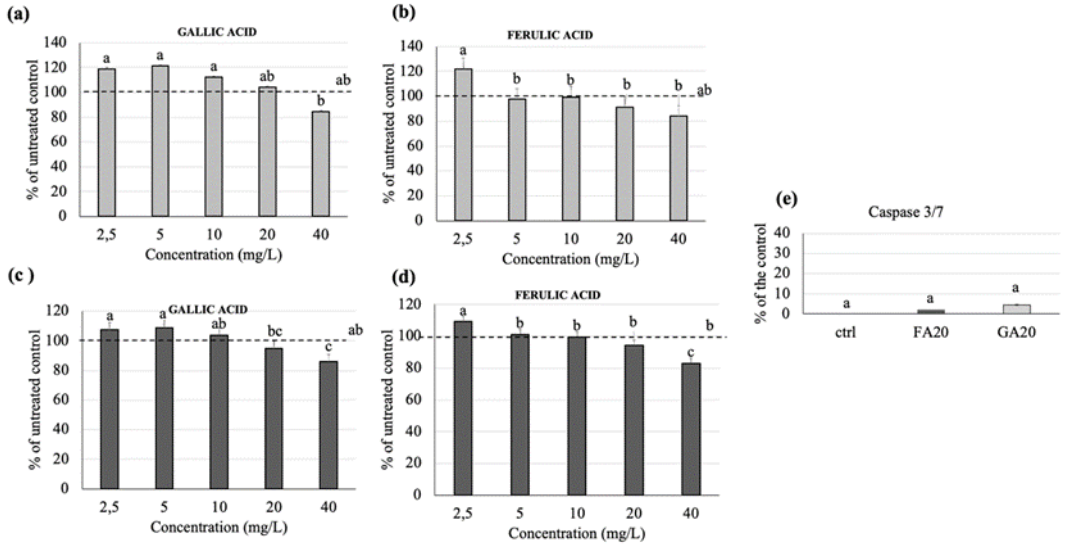

Figure 1. Gallic and ferulic acid effects on L929 mouse fibroblast proliferation and viability. L929 fibroblasts were treated with gallic and ferulic acids at 2.5, 5, 10, 20 and 40 mg/L, respectively. The MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) assay (a,b) and viability assays (c,d) were performed 24 h after treatment. For caspase 3/7 analysis, L929 cells were treated with gallic and ferulic acid at the concentration of 20 _g/mL or diluent as the control. After 24 h, cells were stained with CellEvent. Caspase-3/7 Green Detection Reagent (e). Data are expressed as a percentage of the control. Statistical analysis was performed and, between brackets, different letters indicate mean values that are significantly different at $p < 0.05$.

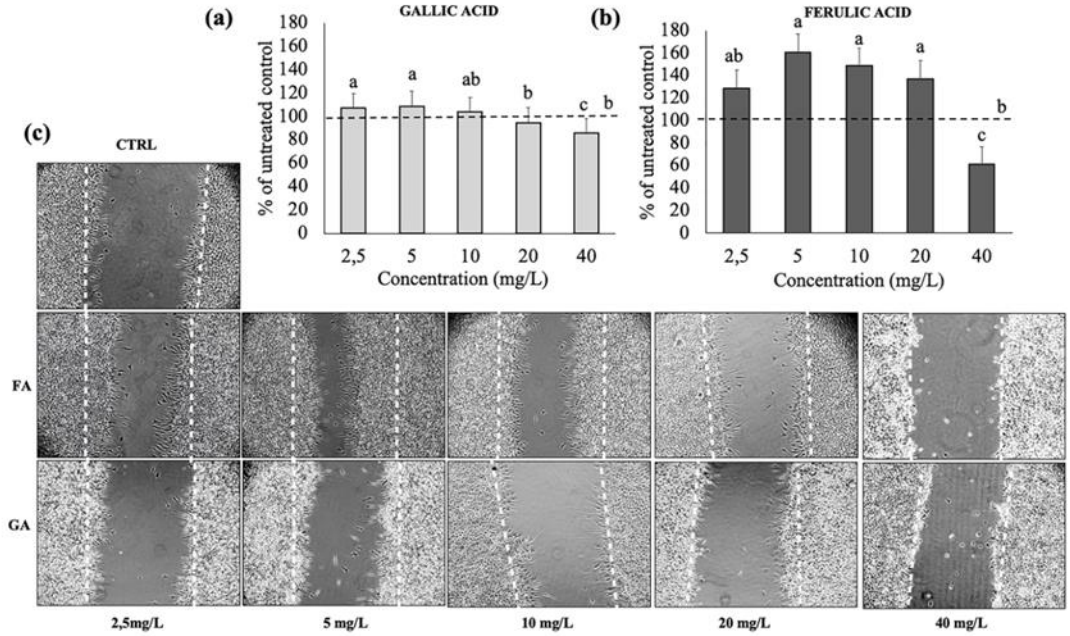

Figure 2. Wound-healing assay using L929 with gallic and ferulic acid. (a) number of fibroblasts migrated after treatment with gallic and (b) ferulic acids at 2.5, 5, 10, 20 and 40 mg/L. (c) Significant pictures of the migrated cells. Data were expressed as percentage compared to the control. Statistical analysis was performed, and between brackets, different letters indicate mean values that are significantly different at $p < 0.05$.

3.3. Effects of Gallic and Ferulic Acid on Human Intestinal Cell Viability

In order to understand the effects of gallic and ferulic acids on the proliferation and viability of cellular components which belong to the intestinal wall mucosa, their effects on the Caco2 colonocyte cell line have been studied. Gallic acid significantly reduced cell proliferation at 40 mg/L (Figure 3a), as well as cell viability at both 20 and 40 mg/L. In contrast, ferulic acid reduced cell proliferation (Figure 3b) and cell viability (Figure 3d) at 10 and 20 mg/L, respectively, but not at 40 mg/L. The quantification of the effector Caspases 3/7, following the treatment of Caco2 with gallic and ferulic acids,at 20 mg/L (Figure 3e) and 40 mg/L (Figure 3f) respectively, for 24 h, showed a significant activation, compared to the control(Figure 4d). An increased effect was elicited by gallic acid.

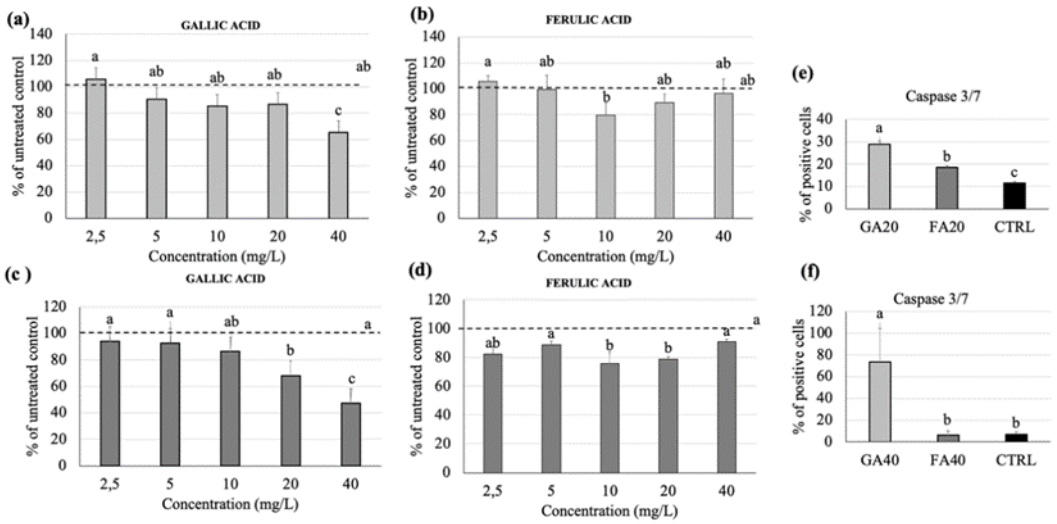

Figure 3. Gallic and ferulic acid effects on Caco2 intestinal cell proliferation and viability. Caco2 cells were treated with gallic and ferulic acids (2.5, 5, 10, 20 and 40 mg/L), respectively. The MTT (a,b) and viability assays (c,d) were performed 24 h after treatment. For caspase 3/7 analysis, Caco2 cells were treated with gallic and ferulic acids at either 20 mg/L (e) or 40 mg/L (f) and diluent as the control. After 24 h, cells were stained with CellEvent. Caspase-3/7 Green Detection Reagent. Data are expressed as a percentage of the control. Statistical analysis was performed, and between brackets, different letters indicate mean values that are significantly different at $p < 0.05$.

3.4. Effects of Gallic and Ferulic Acids on Human Monocyte Cell Viability

To understand the effects of gallic and ferulic acids on the viability of the immunity cellular components of the intestinal dermis compartment, their effects on the U937 monocyte cell line have been studied. Interestingly, gallic acid significantly up-regulated monocyte proliferation at 5, 20 and 40 mg/L, compared to the untreated control (Figure 4a). Cell viability remained unchanged, and was 100% upon each treatment analyzed (Figure 4c). Ferulic acid had no significant effect on U937 cell proliferation, with the exception of the highest dose, namely 40 mg/L (Figure 4b). Cell viability was similarly unchanged at all concentrations analyzed (Figure 4d). The quantification of Caspases 3/7, after cellular treatment with gallic and ferulic acids, respectively, showed no significant difference compared to the untreated control (Figure 4e. The potential effects of gallic and ferulic acids (20 mg/L, respectively) on the migration of human monocytes were studied using Boyden chambers, in either the presence or absence of Caco2 cells (Figure 4f,g). The number of migrated cells were statistically higher in the presence of Caco2 with gallic and ferulic acids, compared to both the untreated control and treated samples without intestinal cells.

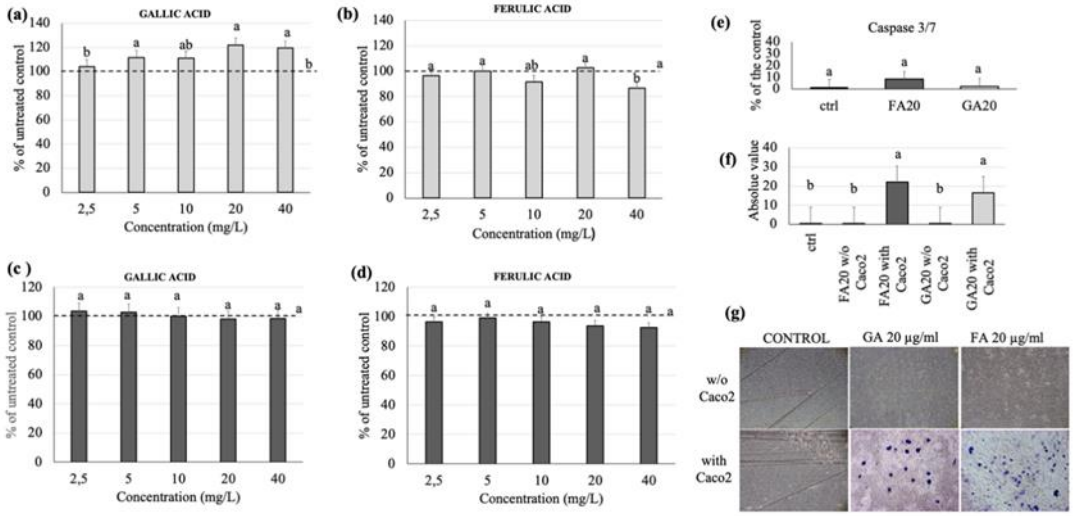

Figure 4. Gallic and ferulic acid effects on U937 monocytes cell proliferation and viability. U937 cells were treated with gallic and ferulic acids (2.5, 5, 10, 20 and 40 mg/L) respectively. The MTT (a,b) and viability assays (c,d) were performed 24 h after treatment. For caspase 3/7 analysis, U937 cells were treated with gallic and ferulic acids, respectively, at 20 mg/L or diluent as the control. After 24 h, cells were stained with CellEvent. Caspase-3/7 Green Detection Reagent (e). U937 cell migration was measured as indicated in the Materials and Methods section, either with or without Caco2 cells. The mean number ± SD of migrated cells were counted and data are expressed as percentage of the control (f,g). Statistical analysis was performed, and between brackets, different letters indicate mean values that are significantly different at $p < 0.05$.

3.5. Linear Discriminant Analysis (LDA)

The entire data set was analyzed by LDA, in order to explain the observed response variability of the investigated cell lines (Caco2, L929, U937) as a function of the treatment with different gallic and ferulic acid doses (0, 2.5, 5, 10, 20 and 40 mg/L). Figure 5a shows a scatter plot of the 10 investigated treatments on the space defined by the first two canonical functions (Root 1, Root 2), accounting for 70% and 23% of the total variability, respectively. The multivariate technique showed high discrimination power as indicated by the Wilks lambda value (0.00114), significant at $p < 0.00001$. The scores of the cases showed that the different treatments were grouped separately. As revealed from the values of canonical functions standardized within variance, the distribution of the cases along the positive branch of Root 1 was strongly influenced by the vitality tests on L929 and U937, the MTT test on L929, and the migration test (values of the first canonical discriminant function equal to 1.35, 0.81, 1.01, 1.21,

respectively) (Figure 5b). The gallic and ferulic acid treatments, between 2.5 and 10 mg/L, were scored in the right quadrant of the scatterplot (positive Root 1, positive Root 2), opposite to the 20 and 40 mg/L treatments on the left quadrant (negative Root1, negative Root 2) of the scatterplot. The untreated control was positioned in the midstream, between the low dose (2.5 – 10 mg/L) and high dose (20 – 40 mg/L) groups, respectively. The high dose group is strictly associated with the negative branch of the first component (influenced by the MTT test on U937, with a value of the first canonical discriminant function equal to –1.01). The score of the cases along the second canonical function (Root 2) was mainly influenced by the vitality of Caco2 (value of the second canonical discriminant function equal to 1.22). In addition, LDA showed that all the high dose treatments were completely discriminated (discrimination percentage of 100% at $p > 0.05$), while the low dose treatments were only partially discriminated (mean discrimination percentage of 88.3% at $p = 0.09$).

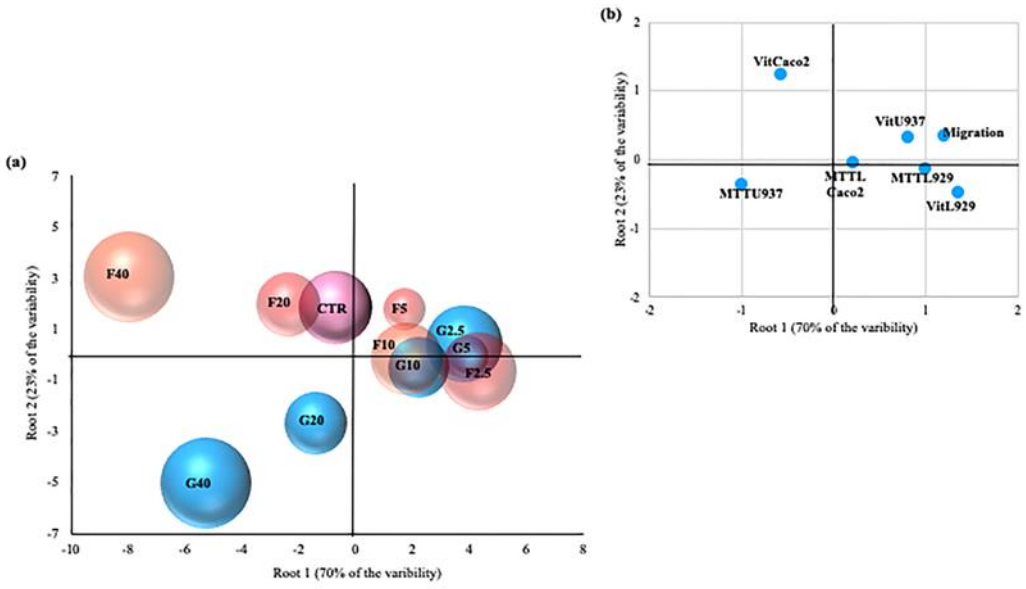

Figure 5. Scatter plot of the 10 investigated treatments, namely (a) gallic and ferulic acids, at doses of 2.5, 5, 10, 20 and 40 mg/L, respectively, which were defined by the first two canonical functions of linear discriminant analysis and (b) projection of variables (as reported in the material and methods section) on the first two dimensions.

3.6. Effect of Gallic and Ferulic Acids on Intestinal Tridimensional Models

The effects of gallic and ferulic acids on the viability and toxicity of the three principal cellular components of the intestinal wall have been studied by using the cell lines individually. To better recapitulate what could happen in the gut wall tissue, an intestinal tridimensional model has been developed. In this model, all three cell populations previously studied were grown together, supported by a collagen type I matrix, able to recreate a support which mimicked the dermis. The tridimensional models were treated for 24 h with both gallic and ferulic acids, at two doses of 5 and 20 mg/L, respectively. Thereafter, tridimensional models were paraffin-embedded and stained (Figure 6). Histological analysis of the mucosal layer showed macroscopic impairment of the mucosal structure, and a down-regulation of occludin expression, with 20 mg/L gallic acid (Figure 6b) compared to the control. In contrast, when using 5 mg/L gallic acid (Figure 6b), there was an up-regulation of the expression of the tight junction occludin (Figure 6c) in treated mucosa, compared to the control. Samples treated with 20mg/L gallic acid significantly down-regulated both the thickness layer and the expression of occludin. No significant effect was elicited by ferulic acid on the epithelial layer structure at either 5 or 20 mg/L (Figure 6b). Again, a decreased expression of occludin was detected in the samples treated with 20 mg/L ferulic acid, compared to the untreated control. No effect on occludin expression was detected with 5 mg/L ferulic acid. A similar effect on occludin expression was observed when intestinal equivalents were performed using the human health cell line NCM460, instead of Caco2 (Supplementary Figure S2). Thiazine staining showed that 20 mg/L gallic and ferulic acids, respectively, induced migration of monocytes in the direction of the epithelial layer, where the integrity was affected.

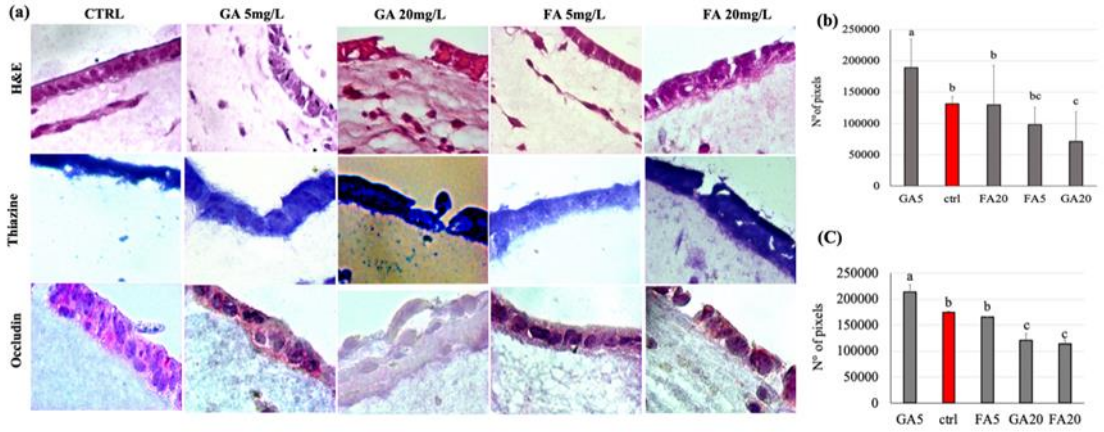

Figure 6. Intestinal equivalents obtained by seeding Caco2 cells on dermal equivalents that were paraffin embedded. (a) Sections were stained with hematoxylin and eosin (H&E), thiazine and occluding markers; Fast Red was used as a chromogen. (b) The thickness of the epithelial layer was measured by image pixel count using ImageJ 2.0.0-rc-69/1.521 (c) Stained areas were evaluated by image pixel count using ImageJ 2.0.0-rc-69/1.521. Experiments were conducted in triplicate from different samples. Statistical analysis was performed, and between brackets, different letters indicate mean values that are significantly different at $p < 0.05$.

4. Discussion

The aim of the present study was to evaluate the effect of two distinct phenolic compounds, using a new in vitro intestinal wall model in order to understand whether polyphenol effects on humans were related to their concentration. While it is well-known that in vitro models are limited for the testing of phenolic compounds, as the latter are mainly metabolized by intestinal microflora, a direct effect of these compounds on intestinal mucosa cannot be excluded. Intestinal microbiota can vary considerably from individual to individual, and may be severely compromised in people suffering from chronic intestinal diseases such as Inflammatory Bowel Disease (IBD) [18]. We tested the effect of gallic acid and ferulic acid on three different cell lines (colonocytes, fibroblasts and monocytes) and on a tridimensional model of intestinal wall tissue, composed of a mucosal layer and a submucosa with fibroblasts and monocytes, respectively. We selected the most represented compound from fruits (gallic acid) and cereals (ferulic acid) for our experiments.

4.1. Gallic and Ferulic Acids and Intestinal Tissue Cell Lines

The present study clearly showed that the differing results obtained were dependent on the cell line used, as well as the polyphenol concentrations. In fibroblasts, no effect

on cell proliferation was elicited by either gallic or ferulic acids at any of the concentrations tested. Moreover, no difference in caspase 3/7 activation was found between treated cells and control cells. Ferulic acid was shown to significantly improve cell viability, whereas no effect was obtained with gallic acid. Interestingly, both polyphenols were toxic when administered at a dose of 40 mg/L. Using the wound-healing assay, it was possible to analyze the capacity of gallic and ferulic acids to induce fibroblasts to heal an in vitro wound. This process is attributable to a precise balance between the migration, proliferation and differentiation of cells near the damaged area [19]. The fibroblasts represent the cell population, which is directly involved in the production of the dermis matrix and has the ability to heal skin wounds [20]. On fibroblasts, ferulic acid was able to promote wound-healing at 5, 10 and 20 mg/L, but inhibited cell migration at the dose of 40 mg/L. Gallic acid stimulation improved wound-healing at 2.5 and 5 mg/L, but similarly induced toxicity at the 40 mg/L dosage. Caco2 cell lines originate from adenocarcinoma, and are the subject of numerous publications promoting their use as biological reference models for the study of enterocytes in vitro [21]. In the present experiments, gallic acid induced toxicity by inhibiting proliferation at 40 mg/L, and had an effect on cell viability starting from 20 mg/L. Instead, ferulic acid was shown to be toxic at a lower dose of 10 mg/L, by inhibiting proliferation. From 10 to 20 mg/L, ferulic acid was toxic as it inhibited cell viability, whilst surprisingly, no effect was detected using the 40 mg/L dose. Moreover, these data were confirmed by caspase 3/7, which showed a similar trend, suggesting that, in Caco2 cells, the down-regulation of proliferation and viability could be mediated by the induction of cell death by activating the apoptotic process. In the intestinal wall tissues are components of innate immunity, such as monocytes and dendritic cells, which are able to respond to oxidative stress and infections [22]. Using the U937 monocyte cell line, gallic acid was shown to up-regulate monocyte activation, starting from the 5 mg/L dose, with no effect on cell viability. Ferulic acid was shown to have no effect on proliferation except at 40 mg/L, and no effect on cell viability was detected. No difference in caspase 3/7 activation was found between treated cells and

control cells. Unlike the other cell lines analyzed, the viability of U937 cells were largely unaffected when treated with the two phenolic acids. This suggests that, as a consequence of the immunity nature of these cells, an inflammatory or stressful stimulus may act to induce a greater proliferation of these cells. To investigate a possible interaction between epithelial cells and monocytes, a migration assay was performed using U937 cells alone, and U937 cells co-cultured with Caco2 cells. Interestingly, cell migration was observed only when U937 and Caco2 were co-cultured. This data suggests that gallic and ferulic acid may induce monocyte migration through the signals released by Caco2 cells, which showed apoptotic activation under stimulation of both polyphenols at 20 mg/L [23].

The LDA analysis showed that, taken together, from all the results obtained from the analysis of the cell lines cultured in the monolayer, it was possible to clearly discriminate all the high dose treatments (20 and 40 mg/L) of both gallic and ferulic acids, which produced negative effects on cells, from the low dose treatments (2.5, 5 and 10 mg/L). The low dose treatments were only partially discriminated, but with overall positive effects on cell health.

4.2. Gallic and Ferulic Acids and Tridimensional Intestinal Equivalent Model

Once the cells were studied individually, the effects of gallic and ferulic acids were tested on a three-dimensional system composed of all three cell lines that were previously analyzed singularly. The three-dimensional cell system, in which several cell lines were assembled to constitute an artificial tissue, represents an alternative method to both in vivo mouse models and in vitro bidimensional cell cultures [24]. Interestingly, a different effect was observed when polyphenols were individually tested in this model. First of all, when analyzed as part of a complex tissue, treatment with 20 mg/L gallic acid induced macroscopic histological damage with lesions similar to ulcerations. These lesions may be related to a significant decrease in the occludin expression observed. Ferulic acid only induced a significant decrease in occludin expression, but both stimuli induced monocyte migration close to the epithelial layer. These effects were not detectable at doses of 5 mg/L. At this concentration, gallic acid seemed to improve intestinal permeability, by increasing epithelial thickness and

occludin expression compared to the control. Basically, the results obtained with the three-dimensional model allowed us to set the toxicity of these compounds at a lower dose than that suggested by tests conducted on monolayer models, which also showed varying responses at different treatments depending on the cell line.

4.3. Conclusions

All these data, taken together, suggested that gallic and ferulic acids can exert toxic effects on the intestinal wall, if present at high concentrations. The concentrations of both gallic and ferulic acids normally consumed in the diet can vary. Sosulski and coworkers [25] found that in flour, directly after milling, the trans-ferulic acid content was 63.6 g/g, while in flour stored for 6 months, the level was 23.3 g/g. Keskin-Šaši´c et al. showed that gallic acid concentration can vary from 7.3 mg GAE/100 mL in aloe vera juice to 71.8 mg GAE/100 mL in cranberry juice [26]. From the present investigation, we found that the two commercially available supplements analyzed displayed concentrations of gallic and ferulic acids that were significantly higher than the amounts present in the respective flour or fruit sources. In the present study, no in vivo assays have been performed, thus it is not possible to predict the real role of the gut microbiome during the absorption mechanism in the intestinal tract for the phenolic compounds analyzed. Moreover, the in vitro intestinal models used in the present work only simulate what happens in vivo. Taken together, our results suggest the idea that high ingestion of polyphenols could induce negative effects on the intestinal wall's integrity. However, more studies, especially with in vivo models and pre-clinical or clinical studies that include the metabolism due to the gut microbiome, are needed to more firmly confirm this hypothesis.

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General Conclusion

Polyphenols have been widely studied for their health benefits, and they are used as supplements improving human health or prevention from some chronic diseases from nutraceutical and biopharmaceutical point of view. From this perspective, the information of metabolism, bioavailability, permeability and solubility regarding 10 dietary polyphenols (ferulic acid, chlorogenic acid, rutin, quercetin, apigenin, cirsimaritin, daidzein, resveratrol, ellagic acid, and curcumin) and their association with the BCS classification were summarized. The BCS classification is generally consistent with the bioavailability data found in the current literature, for example, ferulic acid attributed to BCS Class II shows fast metabolism and excretion rate and non-dose-dependent peak plasmatic concentration, although there are controversies and mismatches of definition of the criteria adopted to establish the administrable dosage.

In chapter 2 and 3, the effects of storage and processing method on the polyphenol content from four wheat cultivars (Verna, Timilia, Sagittario and Claudio) were investigated. Most of the analyzed parameters illustrated differences in cultivars. The whole wheat flour and sprouts of Verna showed higher levels of polyphenol and antioxidant activity than Timilia, Sagittario, and Claudio. However, the microgreens of Claudio showed higher value of polyphenol content and antioxidant potential than other three cultivars. The polyphenol content of whole wheat flour was not significantly affected by the two storage conditions (air and vacuum conditions). There was a decrease of total polyphenol content of wheat flour observed after storage for 8 weeks. Regarding the phenolic acids of wheat flour, either decrease or increase of content were shown, but no compound was completely degraded during storage. The polyphenol content of wheat sprout was generally not significantly affected by the two different light conditions (LED and FL light). While the polyphenol level in some specific species of wheat microgreens was partially influenced by different lights, the total polyphenol and flavonoid contents were significantly increased for wheat microgreens grown under LED light, when compared to fluorescent light.

In chapter 4, the potential of ferulic acid and gallic acid developing as supplement

was evaluated. The toxic effect of ferulic and gallic acids on viability, proliferation and migration of three different cell lines (Caco2, L929 and U937) were observed if present at high concentrations, which suggested that intake of ferulic and gallic acids with high-doses can cause toxic effects on the intestinal wall.

Overall, our results firstly demonstrated that the polyphenol content and phenolic acids profile of whole wheat flour were significantly affected by the storage time. It will be useful in determining the appropriate length of storage with maintaining its bioactive compounds. Moreover, our results obtained in this study also suggested that LED light is optimal culture condition to produce wheat microgreens with high level of phytochemicals. However, further research, especially the different proportion of red and blue LED light, are required to choose the ideal growth condition for wheat microgreens to develop as a new functional food. Our results also suggested the selection of cultivar for producing whole wheat flour product or wheat microgreens when consumed in daily life. In addition, the results in present study provided more information from *in vitro* intestinal models on the safety dose for daily intake of ferulic and gallic acids, but more studies, especially with *in vivo* models and clinical studies are needed when they are developed as supplements.