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Polyphenol-Rich Foods for Human Health

Edited by
Patrizia Restani

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About the Editor

Patrizia Restani graduated in Pharmaceutical Chemistry and Technology and obtained a PhD in Toxicology from the Università degli Studi di Milano. She is a Full Professor in Food Chemistry at the School of Pharmacy, Università degli Studi di Milano, where she is responsible for teaching: (1) food chemistry; (2) dietetic products; (3) analytical methods for the detection of xenobiotics in foods. She is the Coordinator of the school in Scienze e Sicurezza Chimico-Tossicologiche dell'Ambiente (Chemical Safety and Toxicological Environmental Sciences), Università degli Studi di Milano. She teaches at the doctorate level in Nutrition Sciences and in different post-doc courses in the area of Food and Nutrition. Prof. Restani is involved in numerous national and international research programs in the fields of food safety, dietetic products, risk and benefit assessment. Prof. Patrizia Restani has managed several scientific projects both as a project coordinator and while being responsible for a research unit. She coordinated the European Project PlantLIBRA (Plant Food Supplements: Level of Intake, Benefit and Risk Assessment) in the context of the 7th EU Framework Program, involving 25 partners distributed across four continents. Prof. Patrizia Restani received the title of Honorary Professor at the Transilvanian University of Brasov (Romania). Prof. Patrizia Restani is a scientific member of the Groups of Experts "Food Safety" and "Consumption, Nutrition and Health" at the OIV—International Organization of Vine and Wine—and is a member of the Italian Delegation (Ministry of Agriculture) at the same organization. She is a member of the Technical Committee for Animal Nutrition and Health—Section for Dietetics and Nutrition established by the Ministry of Health. She was a member of two EFSA working groups. She is involved as a Technical adviser and Expert witness for Italian Courts in trials on food supplements and food adulteration and collaborates with public institutions (NAS, Courts) in the analysis of seized products to protect the health of consumers. Patrizia Restani has authored/co-authored more than 360 papers, published in international and national journals/books; more than 150 are peer-reviewed.

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Editorial

Polyphenol-Rich Foods for Human Health

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In recent decades, foods rich in polyphenols have received great interest from researchers, who have performed numerous studies in in vitro and animal models and clinical trials. In parallel, food industries have spent many resources in the formulation of functional foods and food supplements that could provide the consumer with an enriched source of these molecules. The consumer has certainly appreciated the expansion of the market for products of "natural" origin, as they consider them (often erroneously) safe by definition. Polyphenols, compounds from vegetable origin with no nutritional value, have long been considered "functional ingredients", i.e., positive for human health. On the other hand, given that polyphenols are not strictly necessary for the physiological functions of the body, as in the case of vitamins and minerals, the scientific committees often raise concerns on the validity of the studies from which any recommendations for intake derive. Among the few claims allowed for polyphenols, there is that published by the EFSA (Commission Regulation (EU) 432/2012) relating to olive oil: "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress. The claim may be used only for olive oil, containing at least 5 mg of hydroxytyrosol and its derivatives (e.g., oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil".

Among the various properties described, phenolic compounds possess the ability to neutralize free radicals; it is therefore thought that their main role may consist in counteracting oxidative stress at the cellular level, and the health claim allowed for olive oil supports it. The antioxidant effect, which is the activity in counteracting the deleterious effects of free radicals, can modulate many of the risk factors responsible for: (1) chronic degenerative diseases, such as tumor diseases, cardiovascular disorders, metabolic syndrome, and dementia; (2) degenerative physiological processes, such as aging.

In more recent times, several authors have published data in support of other beneficial activities related to polyphenols [1]: anti-inflammatory activity, inhibition of tumor cell proliferation, inhibition of cholesterol absorption, modulation of some enzymatic activities that fall within in the mechanisms of stimulation or inhibition of cellular metabolic processes. On these activities, however, apart from the anti-inflammatory activity [2], there are still many uncertainties. In general, polyphenols are ideally considered and often even advertised as "protectors" easily obtainable with the diet. Numerous authors confirm this hypothesis, but, as mentioned above, most of the studies derive from experimental assays performed in vitro or in animal models, from which extrapolation to humans is difficult. In vitro studies use generally purified molecules at high concentrations unreachable with the dietary intake; even a compound with very high antioxidant activity or other biological activity in vitro could have little or no effect if in vivo it does not reach the target tissue at a sufficient concentration.

Furthermore, the metabolic processes that polyphenols undergo in the human body are not yet fully known, even taking into account that they are consumed with complex matrices (such as fruit and vegetables) or foods in combination. For example, the study by Serafini and co-workers [3] showed how the addition of milk can drastically reduce the antioxidant activity of green and black tea. The effect on the human body may be different from that observed in the tissues in culture, where the molecules are poorly biotransformed. The paper published in 2013 by Stockley and co-workers [4] has

collected and critically evaluated the studies relating to the bioavailability of the various classes of polyphenols taken with the diet and in particular with wine. The review led to the conclusion that there are still many critical issues to be studied before being able to reliably associate the presence of one or more classes of polyphenols in foods with the health effects on the cardiovascular system (the most described one) or on other systems/organs.

One of the few certainties lies in the numerous epidemiological studies, where the consumption of fruit and vegetables is considered critical for the positive effects of the diet on health. In confirmation of this, the Mediterranean diet is now universally known thanks to its inclusion by UNESCO in the intangible heritages of humanity. This Special Issue of *Nutrients* collects new information on the role of polyphenols in human health considering the different classes of molecules, their bioavailability, the synergy between the different active components, and the different food sources. Particular interest was addressed to the metabolic syndrome, which is considered among the main causes of morbidity and mortality of populations both in industrialized areas and in developing countries. The authors have proposed both original studies and reviews of the literature data both reiterating the role of bioavailability (already mentioned above) and identifying the components that have been most active in the diseases considered (in addition to the metabolic syndrome in the various forms, Crohn's disease, and anxiety disorders). The sources of polyphenols described include both plants already studied and appreciated for their beneficial activities (*Vaccinium macrocarpon* or cranberry; *Zea mais* or pigmented corn; *Tilia tormentosa* or linden) and plants or fruits less known in European culture but with promising future development (riceberry from Thailand, Maqui, etc.).

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Article

Anti-Obesity Effect of an Ethanol Extract of Cheongchunchal In Vitro and In Vivo

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Abstract: Cheongchunchal (CE) is a developed crop more highly enriched in cyanidin-3-O-glucoside chloride (anthocyanin) than conventional waxy corn. Anthocyanin has been proven to have anti-oxidant, anti-inflammatory, anti-obesity, and anti-cancer effects. In this study, using high-performance liquid chromatography (HPLC), Cheongchunchal was confirmed to contain 8.99 mg/g anthocyanin. The inhibitory effect of an ethanol extract of Cheongchunchal (CE) on adipocyte differentiation was demonstrated using Oil Red O staining and a triglyceride assay. By conducting Western blotting, we also confirmed the regulatory effect of CE on adipocyte differentiation factors by assessing changes in the levels of factors that play a significant role in the differentiation of 3T3-L1 preadipocytes. A C57BL/6N mouse model of obesity was induced with a high-fat diet, and CE (400, 600, and 800 mg/kg/day) or Garcinia (245 mg/kg/day) was orally administered to verify the anti-obesity effect of CE. As a result of CE administration, the food efficiency ratio (FER), weight gain, and weight of tissues decreased. Additionally, blood biochemical changes were observed. Furthermore, the inhibitory effect of CE on adipocytes was confirmed through morphological observation and the expression of adipocyte differentiation-related factors in the liver and fat tissues. Therefore, in this study, we verified the anti-obesity effects of anthocyanin-rich CE both in vitro and in vivo.

Keywords: anti-obesity; Cheongchunchal; anthocyanin; 3T3-L1 preadipocytes; C57BL/6N model

1. Introduction

Obesity is defined as the accumulation and lipid filling of adipocytes that occur under conditions of nutritional imbalance or when the energy intake exceeds the energy consumption [1,2]. It causes many complications, such as a high blood pressure, diabetes, fatty liver, cardiovascular disease, and hyperlipidemia because of metabolic abnormalities. Additionally, obesity increases the possibility of developing osteoarthritis and back pain from excessive weight [3–5]. Many treatments to address the issue of obesity, including exercise, diet therapy, medication, and surgery, have been introduced [6]. However, although some obesity drugs in use are effective in inducing weight loss, their side effects limit their use as a treatment [7,8]. As a result, researchers are actively developing anti-obesity drugs using natural substances, since they have fewer side effects [9–11].

Obesity occurs when preadipocytes in the body are induced to differentiate, the cell cycle stops, and the number of mature adipocytes suddenly increases [12,13]. Recent studies have found that some

nutrients, dietary fibers, and phytochemicals in plants inhibit differentiation into mature adipocytes. Consequently, products related to body fat reduction with these functional food ingredients are being developed [14–16]. Conventional waxy corn is a crop with a recessive gene—wx (waxy)—that has been popular as a snack in Korea for a long time. It is relatively easy to cultivate, and a stable cultivation area is being maintained because of the development of excellent, high-quality varieties of waxy corn; a strong market preference for well-being foods; and the development of various processed products [17,18]. Anthocyanins, which are known to possess anti-oxidant, anti-inflammatory, anti-obesity, and anti-cancer effects, are found in many crops, such as purple corn, black beans, and purple sweet potatoes.

Recently, a variety of anthocyanin-rich crops have been developed [19–21]. If conventional waxy corn were to be used to develop an anthocyanin-rich crop, it could be sold as green corn that could be directly consumed, and would therefore increase in value. The Cheongchunchal used in this study is a type of corn that was developed to contain large amounts of anthocyanins. The anti-obesity effects of various plants and compounds have been confirmed in many studies by investigating the inhibition of preadipocyte differentiation into mature adipocytes in vitro or the factors related to weight, tissue weight, and fat differentiation in in vivo animal models of obesity. Therefore, to confirm the anti-obesity effect of Cheongchunchal, which is an anthocyanin-rich variety of waxy corn, we studied the inhibition of differentiation in vitro in 3T3-L1 preadipocytes through a WST-1 assay, Oil Red O staining, triglyceride analysis, and Western blotting. Moreover, body fat suppression and obesity-related factors were investigated using weight gain, measured tissue weights, and the results of blood biochemical tests of the C57BL/6N model of obesity based on a high-fat diet, in order to show the anti-obesity effect of Cheongchunchal.

2. Materials and Methods

2.1. Reagents

The Cheongchunchal (Grant number: No. 6278) used in this experiment originated from Hongcheon, Gangwon-do, Korea, and the plant is a variety developed in Korea that is scheduled to be listed as an international plant resource. The listing information will be disclosed through future research. The Cheongchunchal cob was dried and crushed. Then, 2000 mL of 40% ethanol was added to 250 g of Cheongchunchal to block light, followed by reflux extraction at room temperature for 6 h. The extracted Cheongchunchal was concentrated under reduced pressure using a vacuum concentrator and then stored at −86 °C. To prepare an ethanol extract of Cheongchunchal (CE) at each concentration examined (200, 400, 800, and 1000 μg/mL), the extract was prepared by dissolving it in an equal volume of dimethyl sulfoxide (DMSO). The prepared samples for each concentration were stored frozen at −20 °C before use.

2.2. Quantitative Analysis of the Cyanidin-3-O-Glucoside Chloride (Anthocyanin) Content in the CE Using High-Performance Liquid Chromatography (HPLC)

The CE was prepared by mixing it with water (Burdick & Jackson, USA) and methanol (Burdick & Jackson) at a ratio of 30:70 (*v*/*v*) to 500 mg/L, followed by stepwise dilution to 1 mg/L. Anthocyanin was also diluted to 500 mg/L with the same solvent and used as a standard stock solution. For quantitative analysis of the anthocyanin content in CE, a standard curve was generated by diluting the anthocyanin to 0.1, 0.2, 0.5, 1, and 2 mg/L, and the analysis was repeated three times. All substances were analyzed by an injection of 10 μL of the sample into Shimadzu HPLC i-Series LC-2030 LT (SHIMADZU, Japan) and separation at a flow rate of 1.0 mL/min through a SunFireTM C-18 column (4.6 × 250 mm, 5 μm, Waters, Germany). In addition, mobile phase A consisted of 0.1% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MI, USA) added to water, and mobile phase B consisted of 0.1% trifluoroacetic acid added to acetonitrile (Burdick & Jackson) (*v*/*v*). The change in the ratio of the mobile phases is shown in

Table 1, and the absorbance at 520 nm was detected using deuterium (D2) lamps (SHIMADZU) over a 35-min period.

Table 1. Change of the ratio of the mobile phase according to the retention time.

Time (min)	Mobile phase A(%) [1]	Mobile phase B(%) [2]
0	90	10
20	75	25
21	0	100
22	0	100
23	90	10
35	90	10

[1] Mobile phase A (%); 0.1% trifluoroacetic acid added to water [2] Mobile phase B (%); 0.1% trifluoroacetic acid added to acetonitrile.

2.3. 3T3-L1 Preadipocyte Cell Culture

The 3T3-L1 preadipocytes were obtained from the American Type Culture Collection (ATCC, MD, USA), and Dulbecco's modified Eagle medium (DMEM) containing 10% bovine calf serum (HyClone Laboratories Inc., Logan, UT, USA) and 1% antibiotics was used as the culture medium. The cells were cultured in an incubator under the conditions of 5% CO_2 at 37 °C. After the cells had been suspended using Trypsin-EDTA (HyClone Laboratories Inc.) every 48 h, the cells were seeded at 1×10^6 cells/mL and subcultured.

2.4. WST-1 Assay

The 3T3-L1 preadipocytes were seeded at 1×10^5 cells/well in a 24-well plate for the cell culture. After incubation for 24 h, the cells were treated with CE at a range of concentrations (200, 400, 800, and 1000 μg/mL) for 48 h. A total of 50 μL of WST-1 solution (Daeillab, Korea) was added to each well and incubated for 2 h under the conditions of 5% CO_2 at 37 °C, after which 100 μL of the medium treated with the WST-1 solution was dispensed into 96-well plates. Then, the absorbance at 450 nm was measured using a FLUOstar Omega (BMG Labtech, Otenberg, Germany).

2.5. Differentiation Induction

The 3T3-L1 preadipocytes were seeded at 1×10^5 cells/mL in a 6-well plate in DMEM medium containing 10% bovine calf serum and 1% antibiotics. After the cells were determined to be confluent, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μg/mL insulin (DMI solution) were added to DMEM containing 10% fetal bovine serum and 1% antibiotics. The medium was mixed and used to treat the 3T3-L1 preadipocytes for 72 h. Then, CE (200, 400, 800, and 1000 μg/mL) and Garcinia (Gar; 200 μg/mL) were used together to treat the cells. Garcinia, which is a substance with proven anti-obesity effects, was used as a positive control in this study. Then, every 48 h, the medium used to culture the 3T3-L1 preadipocytes was replaced with medium containing 1 μg/mL of insulin with CE (200, 400, 800, and 1000 μg/mL) or Garcinia (Gar; 200 μg/mL) for 8 days. The N group did not induce adipocyte differentiation and did not treat any substances.

2.6. Oil Red O Staining

The 3T3-L1 preadipocytes were seeded at 1×10^5 cells/mL in a 6-well plate, and adipocyte differentiation was induced by treatment with a DMI solution and CE (200, 400, 800, and 1000 μg/mL) or Garcinia (Gar; 200 μg/mL). After adipocyte differentiation, the cells were fixed with 10% formalin and treated with 60% isopropanol. The cells were then stained with an Oil Red O staining solution for 10 min. After washing with DW and photography using a phase contrast microscope (×100), the fat was extracted using 100% isopropanol, and the absorbance at 500 nm was measured.

2.7. Triglyceride Assay

A triglyceride quantification assay kit (Abcam, Cambridge, UK) was used to measure the amount of triglycerides that accumulated in the cells during adipocyte differentiation. The 3T3-L1 preadipocytes were seeded at 1×10^5 cells/mL in a 6-well plate, and adipocyte differentiation was induced by treatment with a DMI solution and CE (200, 400, 800, and 1000 μg/mL) or Garcinia (Gar; 200 μg/mL). After the differentiation-induced cells had been homogenized using a 5% NP-40 solution, the reaction was repeated at 90 °C for 5 min and then at room temperature for 5 min to dissolve the triglycerides. The insoluble material was removed using a centrifuge. After the dissolved triglycerides were diluted 10-fold, lipase was used to treat the triglycerides for 20 min, after which a triglyceride probe and the triglyceride enzyme mix were reacted at room temperature for 60 min. The light intensity at a wavelength of 570 nm was measured using a spectrophotometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

2.8. Western Blotting

Western blotting was performed to confirm the expression of a protein that plays a key role upon the induction from preadipocytes to adipocytes. The 3T3-L1 preadipocytes were seeded at 1×10^5 cells/mL in a 6-well plate, and adipocyte differentiation was induced by treatment with a DMI solution and CE (200, 400, 800, and 1000 μg/mL) or Garcinia (Gar; 200 μg/mL). A total of 150 μL of RIPA lysis buffer (ForBioKorea, Korea) containing 1× phosphatase inhibitor cocktail was added to the differentiated cells in each well to separate the proteins, and the cells were then centrifuged at 14,000 rpm for 20 min at 4 °C, after which the supernatant was removed. In the case of the tissue, it was treated with RIPA lysis buffer containing 1× phosphatase inhibitor cocktail, sonicated, and then centrifuged in the same manner to obtain the supernatant. The extracted protein was quantified by measuring the absorbance at 595 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Then, electrophoresis was performed using an 8% or 10% acrylamide gel, after which the proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 2 h by treatment with 4% bovine serum albumin (BSA), after which antibodies against the following were applied at 4 °C overnight: Peroxisome proliferator-activated receptors (PPARγ); C/EBPα (Santa Cruz Biotechnology, Dallas, USA); Acetyl-CoA Carboxylase (ACC); p-AMPK; and β-actin (Cell Signaling Technology, Danvers, USA). Secondary antibodies were incubated with the membrane at 4 °C for 2 h, and the signal was observed using a UVITEC gel imaging system (Philekorea, Gyeonggi-di, Korea).

2.9. Experimental Animal Breeding and Diet

The experimental animals were 5-week-old male C57BL/6N mice obtained from ENVIGO (Indiana, USA). After adaptation to the environment for 1 week, six animals were randomly divided into six groups (normal-fat diet (NFD), high-fat diet (HFD), Garcinia 245 mg/kg/day (Gar), CE 400 mg/kg/day (400), CE 600 mg/kg/day (600), and CE 800 mg/kg/day (800)). The experimental animals were allowed to freely consume water and food, and mice in the NFD group consumed a normal-fat diet (10% fat kcal) purchased from Purina (Korea). Mice in the other five groups (HFD, Gar, 400, 600, and 800) consumed a high-fat diet (60% fat kcal) purchased from ENIGO. The feed composition is shown in Table 2. The specific experimental conditions of a temperature of 23 ± 2 °C, humidity of 50 ± 5%, and light/dark cycle of 12 h were maintained. Obesity was induced by supplying a high-fat diet for 9 weeks, and at the same time, Garcinia (245 mg/kg/day) and Cheongchunchal (400, 600, and 800 mg/kg/day) were administered orally every day. All animal experiments were conducted with the approval of the Hannam University Animal Experimental Ethics Committee (Daejeon, Korea).

Table 2. Composition of experimental diets.

Ingredient (g/kg)	Normal-Fat Diet	High-Fat Diet
Casein	200	265.0
L-cysteine	3	4.0
Corn starch	150	-
Maltodextrin	-	160.0
Sucrose	500	90.0
Cellulose	50	65.5
Soybean Oil	50	30.0
Lard	-	310.0
Mineral mixture	35	48.0
Vitamin mixture	10	21.0
Choline Bitartrate	2	3.0
Energy (kcal/g)	4	5.1
Blue Food Color	-	0.1
Protein (% kcal)	20	18.3
Carbohydrate (% kcal)	64	21.4
Fat (% kcal)	16	60.3

2.10. Body Weight and Feed Efficiency Measurements

Body weight was periodically measured once a week to observe the condition of the animals during the experiment. Food was supplied daily, and the weight was measured once a week. After 24 h, the amount of feed remaining was measured, and the food intake was calculated as the difference between the weight of the supplied feed and the weight of the remaining feed. The food efficiency ratio (FER) was calculated by dividing the amount of weight gained by the amount of feed consumed during the experiment.

2.11. Blood and Tissue Collection and Analysis

Before the end of the experiment, the experimental animals were sacrificed after fasting for at least 12 h, and blood was collected from the abdominal vein after the abdomen was opened. The collected blood was reacted at room temperature for 30 min and then centrifuged at 14,000 rpm for 20 min to separate the serum. The concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, triglycerides, total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol in the collected serum were measured using a biochemical analyzer (AU480 Chemistry Analyzer, Beckman Coulter, USA). In addition, a rat/mouse insulin ELISA kit (Merck Millipore, Darmstadt, Germany) was used to measure the insulin in the serum. A kit (Adiponectin (mouse) total, HMW ELISA, ALPCO Diagnostics, Salem, USA) was used to measure adiponectin, and a mouse leptin ELISA kit (Merck Millipore, Darmstadt, Germany) was used to measure leptin in the serum. After blood collection, the liver, abdominal fat, kidneys, epididymal fat, visceral fat, and subcutaneous fat were extracted in that order. Parts of the abdominal fat, epididymal fat, and liver tissue samples were fixed in 10% neutral formalin for 48 h. After fixation, a paraffin block was prepared and stained with hematoxylin and eosin (H&E). The stained tissue was observed with an optical microscope at ×100. The remaining tissues were stored at −86 °C for Western blotting.

2.12. Statistical Analyses

The results of the experiments are expressed as the mean and standard error and were analyzed using the SPSS 21.0 program (IBM-SPSS, USA). The statistical significance of the differences between groups was analyzed using a *t*-test. All experiments were repeated three times, and the significance of the differences between groups was verified at the levels of $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Qualitative Analysis of Anthocyanin in CE by HPLC

Anthocyanin and CE were analyzed through HPLC to determine the anthocyanin content in the CE (Figure 1A,B), and anthocyanin solutions at 0.1, 0.2, 0.5, 1, and 2 μg/mL were prepared as standard solutions from which a standard curve was created. The anthocyanin content in the CE was also measured (Figure 1C), which revealed that the CE contained 8.99 mg/g anthocyanin.

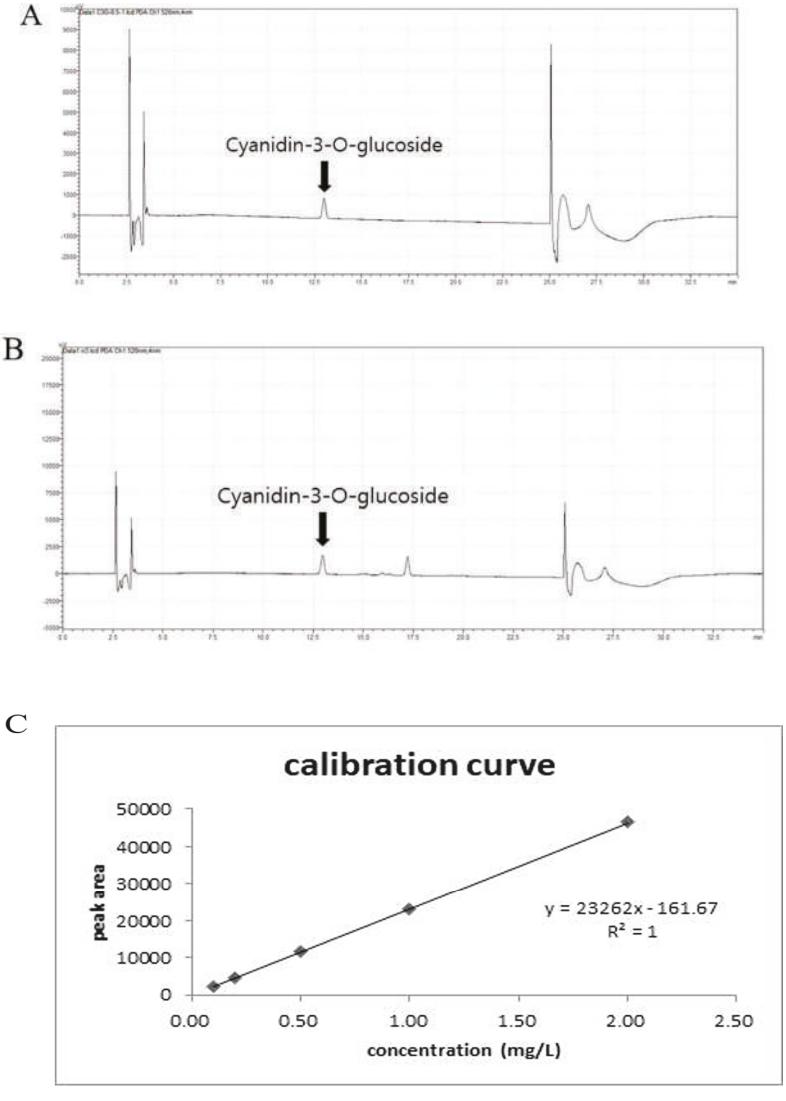

Figure 1. The qualitative identification of anthocyanin in Cheongchunchal (CE). (**A**) High-performance liquid chromatography (HPLC) profiles of CE at 1 mg/L. (**B**) HPLC profiles of anthocyanin at 1 mg/L. The *X*-axis represents the retention time (min), and the *Y*-axis represents the absorption units (μV). (**C**) Standard curve of anthocyanin at 0.1, 0.2, 0.5, 1, and 2 μg/mL. The detector was set at 520 nm.

3.2. Confirmation of the Cytotoxic and Inhibitory Effects on Adipocyte Differentiation

To confirm the cytotoxicity of CE in the 3T3-L1 preadipocytes, the 3T3-L1 preadipocytes were treated with CE at different concentrations (200, 400, 800, and 1000 μg/mL) for 48 h, after which cytotoxicity was confirmed through the WST-1 assay. As a result, the CE was confirmed to exhibit no toxicity in the 3T3-L1 preadipocytes, because the cell viability was more than 90% upon treatment with CE at all concentrations (Figure 2A). The cells were treated with CE at each concentration examined (200, 400, 800, and 1000 μg/mL) or with Garcinia (Gar; 200 μg/mL) and DMI to confirm the effect of inhibiting the differentiation of adipocytes. The degree of adipocyte differentiation in each group was confirmed using Oil Red O staining, which stains hydrophobic components, such as triglycerides, cholesterol, and phospholipids. As a result, the number and size of the differentiated adipocytes were visually confirmed to be significantly decreased by CE or Gar compared to DMI (negative control) (Figure 2B). In addition, the Oil Red O staining intensity in the adipocytes from the different groups was compared. Compared to the N group, the staining intensity was significantly increased in all groups, but a comparison of the staining intensity with that of the DMI group confirmed the CE concentration-dependent reduction in staining. Among the groups, only those administered 800 and 1000 μg/mL of CE showed significantly decreased staining. This effect was most pronounced when the 1000 μg/mL CE-treated group was compared to the Gar group, which acted as the positive control group (Figure 2C). Therefore, the effect of CE on triglyceride accumulation in cells during adipocyte differentiation was confirmed. As a result, triglycerides were significantly increased in all groups except for the 1000 μg/mL CE-treated group compared to the N group, and were significantly decreased in all groups except the 200 μg/mL CE-treated group compared to the DMI group. In addition, the triglyceride content decreased, depending on the CE concentration (Figure 2D). Therefore, concentration-dependent adipocyte differentiation and the inhibitory effects of CE on triglyceride accumulation were confirmed.

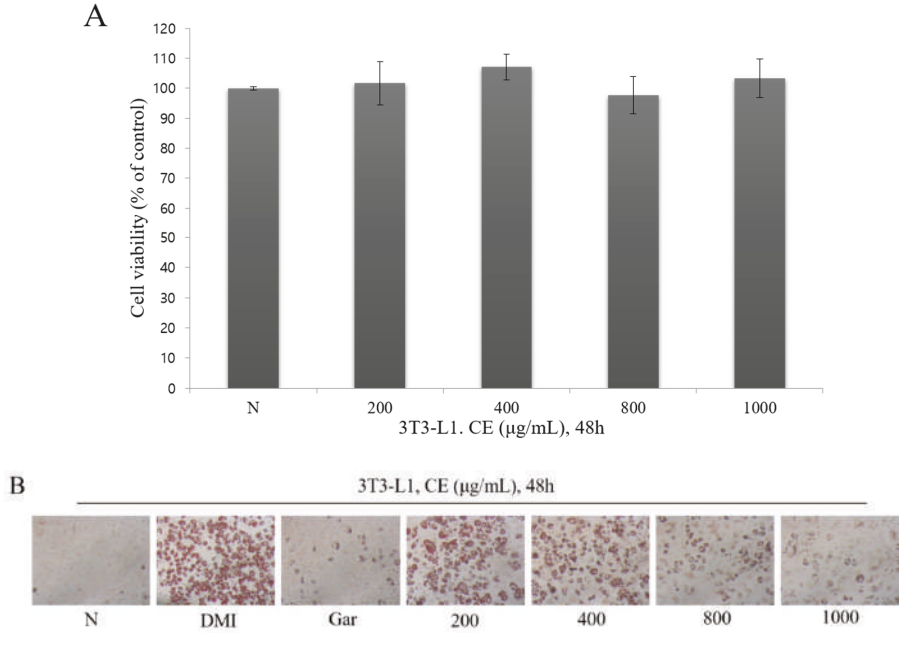

Figure 2. *Cont.*

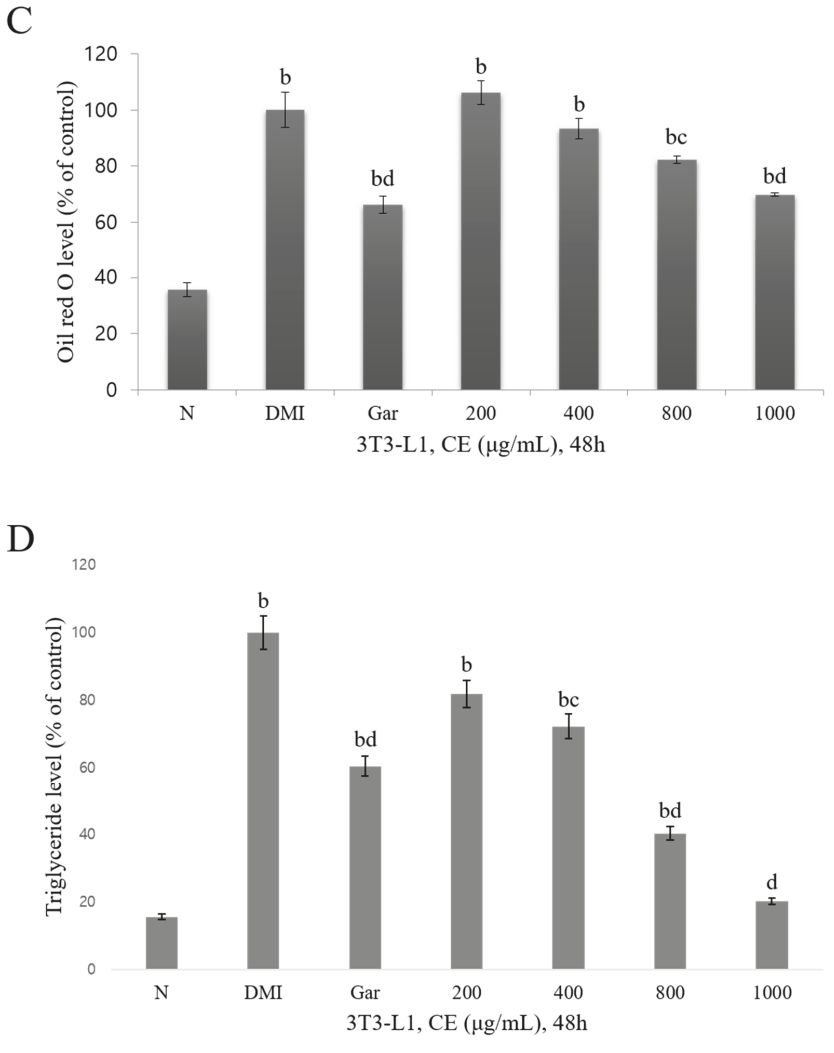

Figure 2. (**A**) The effect of an ethanol extract of Cheongchunchal (CE; 200, 400, 800, and 1000 μg/mL) and Garcinia (Gar; 200 μg/mL) on the cell viability of 3T3-L1 preadipocytes. Cell viability was measured by the WST-1 assay. (**B**) The number and size of differentiated adipocytes were observed with an optical microscope at ×100 (Axiovert 100, Germany). (**C**) Lipid levels (Oil Red O levels) were measured by a spectrophotometer at 450 nm. (**D**) Inhibitory effects of CE on the intracellular triglyceride in 3T3-L1 preadipocytes. The statistical analysis was carried out by the use of a *t*-test. ^{b} $p < 0.01$ compared with group not treated with any substance (N group). ^{c} $p < 0.05$ and ^{d} $p < 0.01$ compared with the 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 1 μg/mL insulin treated group (DMI group). The error bars represent the standard error.

3.3. Confirmation of CE-Induced Changes in the Expression of Proteins Related to Adipocyte Differentiation

When adipocyte differentiation was inhibited by CE (400, 800, and 1000 μg/mL) or Garcinia (Gar; 200 μg/mL) at each concentration tested, changes in the expression of the factors that play a key role in differentiation were confirmed. PPARγ, which is a factor that is mainly expressed in adipose

tissue, promotes the differentiation of adipocytes, and C/EBPα expression is induced during late adipocyte differentiation. In addition, ACC is an enzyme that converts acetyl-CoA to malonyl-CoA and initiates the biosynthesis of fatty acids and triglycerides. PPARγ, C/EBPα, and p-ACC were observed to be decreased in all of the CE-treated groups compared to the DMI group, and CE exerted similar or better effects in all CE-treated groups when compared to the N and Gar groups. In addition, the level of AMPK, which catalyzes the process of ATP production, increases lipolysis in adipose tissue, and decreases fat synthesis, was increased by CE when compared to that in the DMI group, and this effect was confirmed to be concentration-dependent. CE at a concentration of 800 μg/mL or higher had similar or better effects than those observed in the N group, and the effects on the AMPK levels in the 1000 μg/mL CE-treated and Gar groups were the most similar (Figure 3). Therefore, CE was confirmed to inhibit or induce differentiation-related factors during its inhibition of adipocyte differentiation.

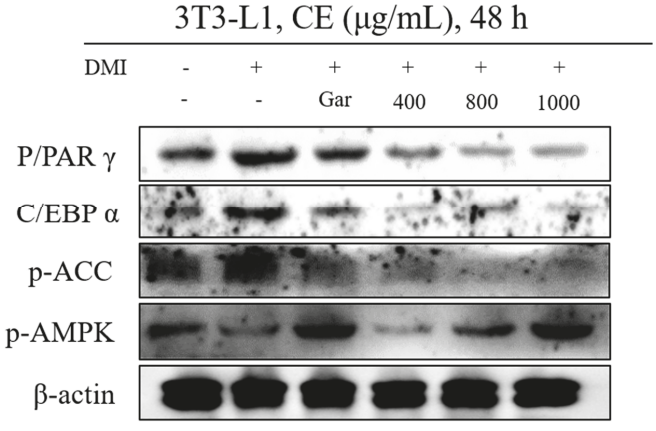

Figure 3. The anethanol extract of Cheongchunchal (CE; 200, 400, 800, and 1000 μg/mL) or Garcinia (Gar, 200 μg/mL) effects on the expression of adipocyte differentiation-related protein. The expression of peroxisome proliferator-activated receptors (PPARγ), C/EBPα, p-ACC, p-AMPK, and β-actin in 3T3-L1 preadipocytes was analyzed by Western blot analysis.

3.4. Confirmation of CE-Induced Changes in the Experimental Animals' Body and Tissue Weights, Food Intake, and FER

The experimental animals were fed a high-fat diet for 9 weeks to induce obesity, and were simultaneously administered CE (400, 600, and 800 mg/kg/day) and Garcinia (Gar; 245 mg/kg/day) orally once a day. All experimental animals were weighed once a week. Weight gain was calculated using the difference in weight at weeks 1 and 9. The results showed increased weight gain in all groups compared to the NFD group, but weight gain was significantly decreased in all CE-treated groups compared to the HFD group (negative control). However, the positive control group (the Gar group) did not exhibit decreased weight gain compared to the HFD group. Through these findings, CE was confirmed to be more effective in suppressing weight gain than Gar. The assessment of food intake showed a decreased food intake in all groups when compared to the NFD group, and the food intake was not significantly decreased in any of the CE-treated groups when compared to the HFD group. The FERs in the CE-treated groups were higher than that in the NFD group and lower than that in the HFD group, but these differences were not significant. Similar changes in food intake and FER were observed in the Gar group. In addition, CE-induced changes in the liver, kidneys, and adipose tissue weight were confirmed. The weights of the liver and kidney tissues were increased in all CE-treated groups compared to the NFD group. However, compared to the HFD group, the 600 and 800 groups showed significantly decreased liver tissue weights, and the kidney tissue weights in the 400 and 800 groups were effectively decreased. CE was found to be more effective in reducing liver and kidney

weights than Gar. The abdominal, epididymal, visceral, and subcutaneous fat weights were increased in all groups compared to the NFD group, but were decreased in all groups compared to the HFD group. However, visceral fat weight was not significantly reduced in any CE-treated group (Table 3). Therefore, these results confirm that CE is effective in suppressing body fat and weight gain.

Table 3. Measurements of weight gain, food intake, the food efficiency ratio (FER), and tissue weight.

Measurements	NFD [1]	HFD [2]	Gar [3]	400 [4]	600 [5]	800 [6]
Weight (g)						
1th weeks (start supplement)	21.00 ± 1.00	21.10 ± 0.75	21.50 ± 0.87	21.48 ± 0.53	21.92 ± 0.13	21.23 ± 0.78
9th weeks (end supplement)	24.63 ± 0.64	37.25 ± 2.17 ^{b}	34.05 ± 1.40 ^{b}	33.48 ± 0.45 ^{b,c}	32.65 ± 2.31 ^{b,c}	32.03 ± 1.36 ^{b,c}
Weight gain (g)	3.63 ± 0.68	16.15 ± 2.44 ^{b}	12.55 ± 1.69 ^{b}	11.98 ± 0.94 ^{b,c}	10.73 ± 2.26 ^{b,c}	10.8 ± 1.47 ^{b,c}
Intake						
Food intake (g/day)	3.21 ± 0.64	2.55 ± 0.43 ^{a}	2.59 ± 0.63	2.32 ± 0.45 ^{b}	2.26 ± 0.35 ^{b}	2.27 ± 0.50 ^{b}
FER [7]	0.018 ± 0.003	0.101 ± 0.02 ^{b}	0.077 ± 0.01 ^{b}	0.082 ± 0.01 ^{b}	0.075 ± 0.02 ^{b}	0.075 ± 0.01 ^{b}
Tissue weight (g)						
Liver	0.93 ± 0.04	1.38 ± 0.14 ^{b}	1.27 ± 0.11 ^{b}	1.23 ± 0.05 ^{b}	1.05 ± 0.06 ^{a,d}	1.06 ± 0.08 ^{a,c}
Kidney	0.31 ± 0.01	0.40 ± 0.01 ^{b}	0.39 ± 0.04 ^{a}	0.37 ± 0.02 ^{b,c}	0.34 ± 0.02 ^{d}	0.35 ± 0.01 ^{b,d}
Adipose tissue weight (g)						
Abdominal fat tissue	0.385 ± 0.08	1.820 ± 0.10 ^{b}	1.283 ± 0.51 ^{a}	1.317 ± 0.08 ^{b,d}	1.211 ± 0.24 ^{b,d}	1.363 ± 0.36 ^{b}
Epididymal fat tissue	0.052 ± 0.01	0.301 ± 0.04 ^{b}	0.141 ± 0.05 ^{a,d}	0.192 ± 0.01 ^{b,d}	0.159 ± 0.03 ^{b,d}	0.168 ± 0.04 ^{b,d}
Visceral fat tissue	0.442 ± 0.08	1.357 ± 0.07 ^{b}	1.117 ± 0.42 ^{a}	1.293 ± 0.07 ^{b}	1.121 ± 0.19 ^{b}	1.215 ± 0.27 ^{b}
Subcutaneous fat tissue	0.411 ± 0.09	2.759 ± 0.12 ^{b}	1.668 ± 0.88 ^{a}	1.860 ± 0.34 ^{b,c}	1.201 ± 0.44 ^{b,d}	1.371 ± 0.51 ^{b,d}

[1] NFD; Normal-Fat Diet. [2] HFD; High-Fat Diet. [3] Gar; Garcinia 245 mg/kg/day. [4] 400; The ethanol extract of Cheongchunchal (CE) 400 mg/kg/day. [5] 600; CE 600 mg/kg/day. [6] 800; CE 800 mg/kg/day. [7] Food Efficiency Ratio (FER) = [weight gain (g/day)]/[food intake (g/day)]. The statistical analysis was carried out by the use of a *t*-test. ^{a} $p < 0.05$ and ^{b} $p < 0.01$ compared with the NFD group. ^{c} $p < 0.05$ and ^{d} $p < 0.01$ compared with the HFD group. Values are the mean ± SD of six mice per group.

3.5. Confirmation of Blood Biochemical Changes Induced by CE

After the collection of blood from all experimental animal groups and serum separation, blood biochemical changes were observed. To confirm the effect of CE on fatty liver disease, changes in ALT and AST were confirmed. Compared to the NFD group, all CE-treated groups showed increased ALT and AST levels, and both factors were decreased in all CE-treated groups compared to the HFD group, but this difference was only significant for the 400 group. Since ALT and AST levels were increased in the Gar group compared to the HFD group, CE effectively inhibited liver toxicity caused by fatty liver to a greater extent than Gar. Changes in serum glucose were observed to determine how CE affects blood glucose control, which is typically inhibited by obesity. Glucose levels were increased in all CE-treated groups compared to the NFD group, but were decreased significantly compared to those in the HFD group. Triglyceride, total cholesterol, HDL cholesterol, and LDL cholesterol levels were observed to confirm changes in the blood induced by CE. In all CE-treated groups, blood triglyceride levels were increased compared to those in the NFD group, and were decreased compared to those in the HFD group, but this difference was only significant for the 400 and 600 groups. The total cholesterol levels in all CE-treated groups were increased compared to that in the NFD group, and were significantly decreased compared to that in the HFD group. HDL cholesterol was observed to be similar in all CE-treated groups, and was significantly increased in all groups compared to the HFD group. LDL cholesterol was increased in all groups compared to the NFD group, but was significantly decreased in all CE-treated groups compared to the HFD group. Therefore, by measuring the biochemical changes in the blood, abnormal hepatotoxicity, blood sugar, and triglyceride and cholesterol levels due to obesity were found to be regulated by CE, but this effect was not concentration-dependent (Table 4).

Table 4. Measurements of blood biochemical changes.

Measurements	NFD [1]	HFD [2]	Gar [3]	400 [4]	600 [5]	800 [6]
ALT (u/L)	39.83 ± 2.78	110.97 ± 19.21 [b]	246.13 ± 94.34 [a,d]	60.30 ± 10.09 [a,c]	90.27 ± 14.18 [a]	69.03 ± 20.34
AST (u/L)	23.33 ± 0.58	116.00 ± 50.48 [a]	136.00 ± 22.54 [a]	37.67 ± 9.07 [c]	76.00 ± 9.64 [a]	75.67 ± 17.10 [a]
Glucose (mg/dL)	220.67 ± 13.05	370.67 ± 7.57 [b]	242.00 ± 24.25 [d]	250.00 ± 33.06 [d]	269.00 ± 56.40 [c]	290.33 ± 40.80 [a,c]
Triglyceride (mg/dL)	84.33 ± 0.58	193.67 ± 7.23 [b]	189.67 ± 4.04 [b]	167.33 ± 9.07 [b,c]	151.33 ± 11.37 [b,d]	169.00 ± 16.52 [b]
Total-cholesterol (mg/dL)	56.67 ± 4.04	116.00 ± 18.03 [b]	79.33 ± 5.13 [b,c]	71.67 ± 1.53 [b,c]	63.00 ± 3.61 [d]	74.33 ± 9.71 [a,c]
HDL-cholesterol (mg/dL)	109.00 ± 2.65	61.67 ± 0.58 [b]	117.00 ± 3.61 [a,d]	115.00 ± 7.55 [d]	106.33 ± 5.03 [d]	109.33 ± 3.21 [d]
LDL-cholesterol (mg/dL)	10.33 ± 0.58	21.33 ± 1.53 [b]	20.00 ± 2.65 [b]	16.00 ± 2.00 [b,c]	15.00 ± 1.73 [a,d]	17.33 ± 0.58 [b,c]

[1] NFD; Normal-Fat Diet. [2] HFD; High-Fat Diet. [3] Gar; Garcinia 245 mg/kg/day. [4] 400; The ethanol extract of Cheongchunchal (CE) 400 mg/kg/day. [5] 600; CE 600 mg/kg/day. [6] 800; CE 800 mg/kg/day. The statistical analysis was carried out by the use of a *t*-test. [a] $p < 0.05$ and [b] $p < 0.01$ compared with the NFD group. [c] $p < 0.05$ and [d] $p < 0.01$ compared with the HFD group. Values are the mean ± SD of six mice per group.

3.6. Confirmation of Changes in the Serum Concentrations of Insulin, Adiponectin, and Leptin

Blood was collected from all experimental animals; the serum was separated; and changes in the levels of insulin, adiponectin, and leptin in the serum caused by CE were confirmed. Obesity induces resistance to insulin and reduces the function of pancreatic beta cells, which secrete insulin. Insulin was increased in the blood of the experimental animals in all groups compared to the NFD group, but this difference was not significant in the 600 and 800 groups. In addition, all groups showed decreased insulin compared to the HFD group (Figure 4A). Adiponectin is a protein that promotes the β-oxidation of fatty acids in muscles and inhibits fat synthesis in adipose tissue. Adiponectin was decreased in all groups compared to the NFD group, but was increased compared to the HFD group. However, this change in adiponectin levels was not significant in any of the groups (Figure 4B). Leptin is a protein secreted by adipocytes; when weight increases, leptin increases, inhibiting food intake and increasing physical activity. The assessment of the level of leptin expression showed that leptin was significantly increased in all groups, except in the Gar group, compared to the NFD group, and was significantly decreased in only the 600 group compared to the HFD group (Figure 4C). Therefore, the effect of CE against obesity was observed through the assessment of the above biomarkers. In addition, insulin resistance caused by obesity was indirectly confirmed to be alleviated by CE, thereby helping to alleviate type 2 diabetes.

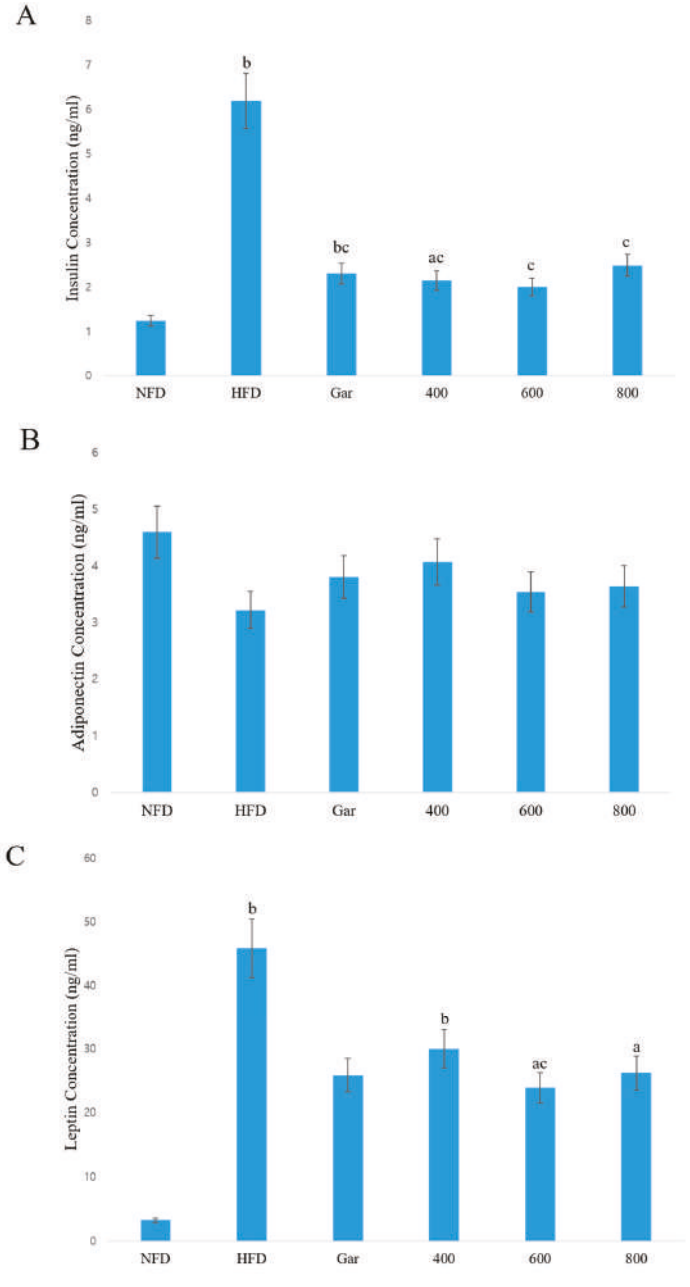

Figure 4. Concentrations of (**A**) insulin, (**B**) adiponectin, and (**C**) leptin in serum were measured by an ELISA kit. NFD; Normal-Fat Diet. HFD; High-Fat Diet. Gar; Garcinia 245 mg/kg/day. 400; The ethanol extract of Cheongchunchal (CE) 400 mg/kg/day. 600; CE 600 mg/kg/day. 800; CE 800 mg/kg/day. The statistical analysis was carried out by the use of a *t*-test. [a] $p < 0.05$ and [b] $p < 0.01$ compared with the NFD group. [c] $p < 0.05$ compared with the HFD group.

3.7. Confirmation of Changes in Liver Morphological- and Adipocyte Differentiation-Related Factors

To assess the morphological changes due to fatty liver, the livers were collected from the experimental animals and subjected to H&E staining before the comparison. The results showed that fatty liver was mostly found in the HFD group, and that fatty liver was decreased in all groups compared to the HFD group (Figure 5A). In addition, trends in the expression of PPARγ, C/EBPα, and p-ACC in the liver tissues of each group were confirmed through Western blotting. The expression of all proteins tended to be decreased compared to the expression in the HFD group. (Figure 5B). Therefore, the inhibitory effect of CE on fatty liver was confirmed through morphological observation and the expression of adipocyte differentiation-related factors.

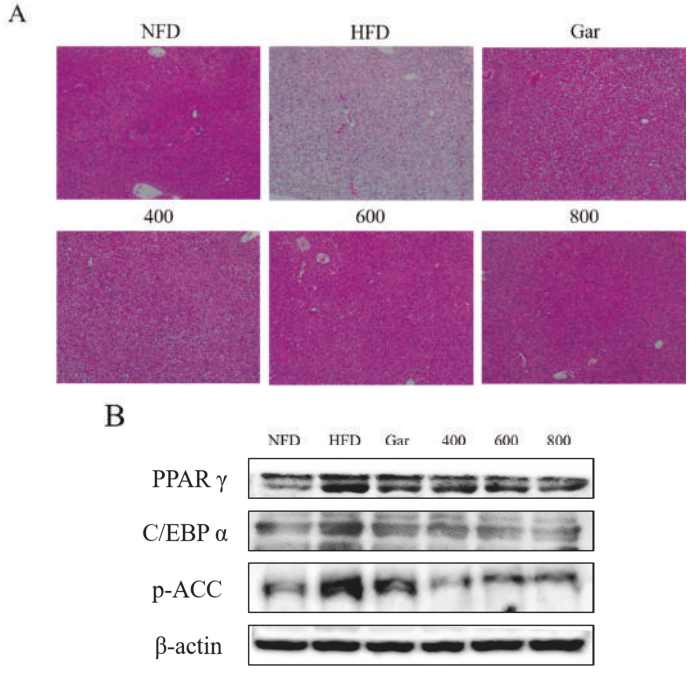

Figure 5. (**A**) Histological change of the liver. Hematoxylin and eosin (H&E) stained sections of liver tissues were observed with an optical microscope at ×100 (Axiovert 100, Germany). (**B**) The expression of PPARγ, C/EBPα, p-ACC, and β-actin in the liver was analyzed by Western blot analysis. NFD; Normal-Fat Diet. HFD; High-Fat Diet. Gar; Garcinia 245 mg/kg/day. 400; The ethanol extract of Cheongchunchal (CE) 400 mg/kg/day. 600; CE 600 mg/kg/day. 800; CE 800 mg/kg/day.

3.8. Confirmation of Changes in the Morphological- and Adipocyte Differentiation-Related Factors of Abdominal and Epididymal Fat

To assess the morphological changes in abdominal and epididymal fat, the abdominal and epididymal fat was collected from the experimental animals and compared through H&E staining. As a result, a large number of large adipocytes in both types of adipose tissue were observed in the HFD group, and the size of adipocytes was found to be decreased in all groups compared to the HFD group (Figure 6A,B). In addition, PPARγ and C/EBPα levels in the abdominal fat and epididymal adipose tissue were confirmed through Western blotting. Both factors in both types of adipose tissue were inhibited in all CE-treated groups compared to the HFD group (Figure 6C,D). Therefore, changes in the levels of adipocyte differentiation factors and a decrease in the size of the adipocytes in adipose tissue were observed to be induced by CE.

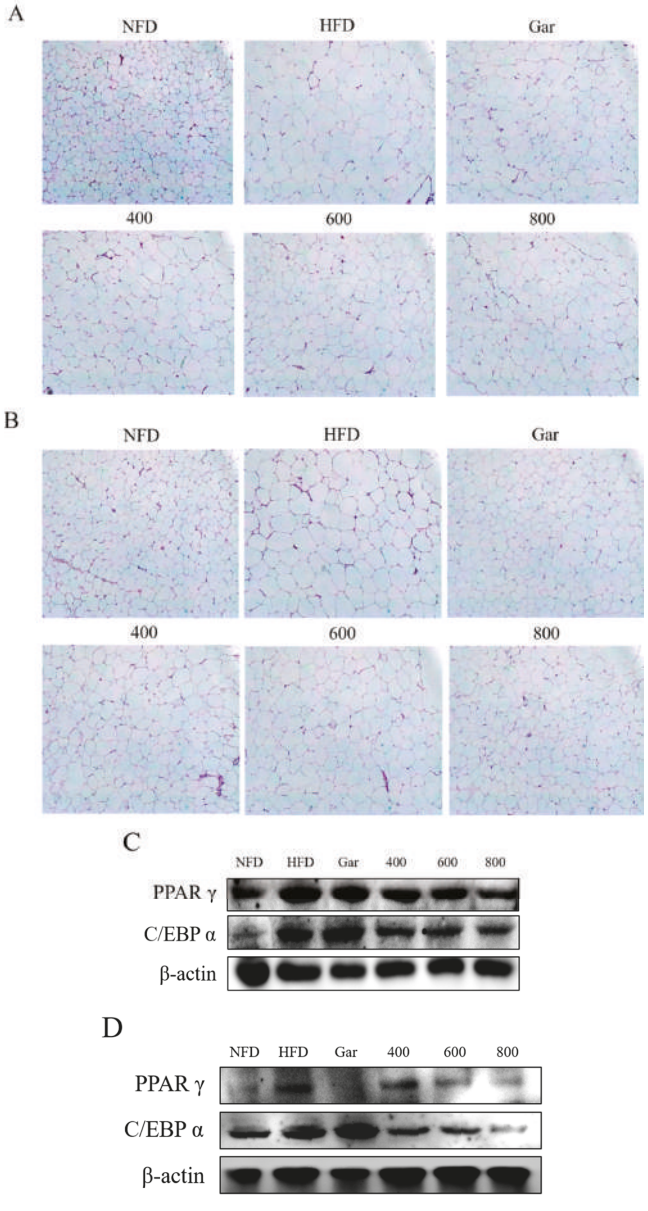

Figure 6. (**A**) Histological change of abdominal fat tissue. (**B**) Histological change of epididymal fat tissue. H&E stained sections of fat tissues were observed with an optical microscope at ×400 (Axiovert 100, Germany). (**C**) The expression of PPARγ, C/EBPα, and β-actin in abdominal fat tissue was analyzed by Western blot analysis. (**D**) The expression of PPARγ, C/EBPα, and β-actin in epididymal fat tissue was analyzed by Western blot analysis. NFD; Normal-Fat Diet. HFD; High-Fat Diet. Gar; Garcinia 245 mg/kg/day. 400; The ethanol extract of Cheongchunchal (CE) 400 mg/kg/day. 600; CE 600 mg/kg/day. 800; CE 800 mg/kg/day.

4. Discussion and Conclusions

Many studies focused on anti-obesity drugs and functional foods are being conducted to address the recently increasing number of obesity cases [22,23]. Additionally, many studies are being conducted to verify the effectiveness of anti-obesity drugs and functional foods with natural substances, as well as to reduce the various side effects of some commercial anti-obesity drugs [24–26]. This study was a basic study aimed at developing anti-obesity drugs and functional foods from natural substances. Cheongchunchal is a waxy corn variety developed to contain a large amount of anthocyanin. Previous studies have confirmed that anthocyanin has various effects, including anti-oxidant, anti-inflammatory, anti-obesity, and anti-cancer effects [27–31]. Therefore, we determined anthocyanin as an indicator component. However, anthocyanins are weak to light, and conditions for stable extraction are required [32]. We established an extraction method stably containing anthocyanins in the CE extraction process through this study. Currently, we are analyzing other components that affect anti-obesity in Cheongchunchal, in order to confirm a more accurate anti-obesity effect of CE. In this study, we quantitatively analyzed anthocyanin in CE using HPLC and estimated the anthocyanin content to be 8.99 mg/g. The cytotoxicity of CE in 3T3-L1 preadipocytes was confirmed by the WST-1 assay, but cytotoxicity was not found at all concentrations examined (200, 400, 800, and 1000 μg/mL). The differentiation of 3T3-L1 preadipocytes into adipocytes was induced by treating them with DMI. Simultaneously, the inhibitory effects of CE (200, 400, 800, and 1000 μg/mL) and Garcinia (Gar; 200 μg/mL) on adipocyte differentiation were examined. Using Oil Red O staining, we quantitatively evaluated the morphological properties of the cells and stained adipocytes. This study revealed that, compared to those in the DMI group, the number and size of adipocytes and the level of Oil Red O staining were decreased according to the concentration of CE. Triglycerides, which accumulate in cells during adipocyte differentiation, were also reduced according to the concentration of CE compared to their levels in the DMI group. Using Western blotting, CE was also found to inhibit differentiation by examining the levels of PPARγ, C/EBPα, ACC, and AMPK, which play a major role in controlling adipocyte differentiation. Therefore, we found that CE inhibited adipocyte differentiation by suppressing or inducing differentiation-related factors when the induction of 3T3-L1 preadipocytes into adipocytes was induced. To confirm the anti-obesity effect of CE in vivo, as well as in vitro, the C57BL/6N model mice were fed a high-fat diet to induce obesity, while CE (400, 600, and 800 mg/kg/day) or Garcinia (Gar; 245 mg/kg/day) was administered once a day. Compared to the HFD group, the groups treated with CE at all concentrations tested showed decreased weight gain. No change in the CE-induced food intake was found. Most of the time, the food intake was high in the HFD group. However, the highest food intake was observed in the NFD group. We think the cause of this is as follows: The normal-fat diet is harder to consume than the high fat diet. For this reason, while experimental animals grind feed, the feed splits and a large chunk of feed sometimes falls out of the feed container. However, as a result of calculating the food efficiency ratio, the highest values in the HFD group, and the lowest values in the NFD group, it was determined that there was no experimental error due to food intake. FER was decreased in the CE-treated groups compared to the HFD group, but the differences were not significant. Based on these results, a high-fat diet with a high caloric density increased the FER, but CE could not be confirmed to reduce the digestive absorption rate or utilization rate of some nutrients. Fatty liver is a condition in which fat makes up more than 5% of the liver's weight and the weight of the liver is increased. Since a significant reduction in fatty liver was observed in the CE groups compared to the HFD group, we concluded that CE has an inhibitory effect against fatty liver. A significant decrease in kidney tissue weight was also observed in the CE groups compared to the HFD group. Furthermore, the weight of abdominal, epididymal, and subcutaneous fat was significantly reduced by CE. Although epididymal fat showed a CE-induced reduction in weight, it was not significant. These results confirm the effect of CE on weight loss and its fat-inhibitory effect. Compared to the HFD group, the CE groups showed decreases in ALT, AST, glucose, triglyceride, total cholesterol, and LDL cholesterol levels in the serum, but the HDL cholesterol level was increased by CE. Additionally, after the levels of insulin, adiponectin, and

leptin were measured, insulin and leptin levels were found to be significantly reduced compared to those in the HFD group. Additionally, although an increase in adiponectin was observed, it was not significant. Via H&E staining, the inhibitory effect of CE on fatty liver was confirmed, as morphological changes in the liver tissues and changes in the levels of PPARγ, C/EBPα, and ACC in the liver tissues caused by CE were found. Through these results, we confirmed the inhibitory effect of CE on fatty liver by changes in the levels of differentiation-related factors. Morphological changes in abdominal and epididymal fat by CE were found through H&E staining, and PPARγ and C/EBPα levels were verified to be decreased in both types of adipose tissue. Consequently, we concluded that CE changed the levels of the differentiation-related factors in the two types of adipose tissue, thus inhibiting adipose tissue production. Based on the above results, this study showed that CE exerts an anti-obesity effect by inhibiting the adipocyte differentiation of 3T3-L1 preadipocytes, reducing the body weight and body fat weight (as confirmed using an obesity induction model), and changing the levels of obesity-related factors. Therefore, this study may serve as a basis to investigate the effectiveness and safety of CE as an anti-obesity drug and functional ingredient. Additionally, by reducing blood fat levels, CE may have a positive effect on various obesity-derived diseases.

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Conflicts of Interest: The authors declare no conflict of interest.

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Use of an Animal Model to Evaluate Anxiolytic Effects of Dietary Supplementation with *Tilia tomentosa* Moench Bud Extracts

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Abstract: Anxiety disorders are common and complex psychiatric syndromes affecting a broad spectrum of patients. On top of that, we know that aging produces an increase in anxiety vulnerability and sedative consumption. Moreover, stress disorders frequently show a clear gender susceptibility. Currently, the approved pharmacological strategies have severe side effects such as hallucinations, addiction, suicide, insomnia, and loss of motor coordination. Dietary integration with supplements represents an intriguing strategy for improving the efficacy and the safety of synthetic anxiolytics. Accordingly, a recent article demonstrated that glyceric bud extracts from *Tilia tomentosa* Moench (TTBEs) exert effects that are consistent with anxiolytic activity. However, the effects of these compounds in vivo are unknown. To examine this question, we conducted behavioral analysis in mice. A total of 21 days of oral supplements (vehicle and TTBEs) were assessed by Light Dark and Hole Board tests in male and female mice (young, 3 months; old, 24 months). Interestingly, the principal component analysis revealed gender and age-specific behavioral modulations. Moreover, the diet integration with the botanicals did not modify the body weight gain and the daily intake of water. Our results support the use of TTBEs as dietary supplements for anxiolytic purposes and unveil age and gender-dependent responses.

Keywords: polyphenols; bud-derivatives; nutraceuticals; supplements; anxiety; mood disorders; mice behaviour; principal component analysis

1. Introduction

Anxiety and stress-related disorders are psychiatric conditions vulnerable to the influence of altered signaling from the gut microbiota [1]. Accordingly, the scientific community suggests that a correct interpretation of the diet and, if necessary, a targeted food supplementation can improve the effectiveness of therapies [2]. Another critical point in the management of anxiety-like disorders is the gender and age susceptibility [3–6]. Reporting data demonstrated that females are more affected by anxiety, and that, currently, both young and old people make wide use of anxiolytic drugs [7,8]. The social impact of anxiolytics consumption generates the need to strongly reduce the power of side effects [9–11]. Consequently, the integration of actual therapy with dietary supplements like botanicals could be recommended in the most fragile patients [12]. Interestingly, bud-derivatives, obtained by macerating meristematic fresh tissues of trees and herbaceous plants, represent a relatively new category of botanicals. In most countries of the EU, bud-derivatives, named also gemmoderivatives or

embryo-extracts, are classified as plant food supplements [13–17]. The genotype of the considered species and varieties, the environmental characteristics of the sampling-sites, the phenological stage of the buds, the applied agrotechniques and the manufacturer practices influence the quality of these preparations [18].

Tilia tomentosa Moench buds are used worldwide for the preparation of tea and dietary supplements. This use is justified by a plethora of potential healthy properties attributed to *Tilia tomentosa* Moench Buds Extracts (TTBEs). TTBEs represent one of the dietary supplements investigated in the FINNOVER project (Innovative strategies for the development of cross-border green supply chains), a European Interreg Alcotra Italy/France project (2017–2020). Interestingly, TTTBEs are considered for their potential anxiolytic effects on the central nervous system (CNS) [19,20], probably due to their phenolic composition mainly represented by flavonols (quercetin, kaempferol, and apigenin derivatives) and phenolic acids.

Recently, Allio and colleagues examined the impact of TTBEs at gamma-aminobutyric (GABA)ergic synapses by performing post-synaptic voltage-clamp recordings in neuronal cultures. Direct application of TTBEs on post-synaptic terminals activated a chloride current in a way consistent with the selective activation of $GABA_A$ receptors [20]. Dysfunctions of the GABA system in the CNS have long been associated with anxiety and mood disorders [21,22]. Similarly, noradrenaline is also known to play a main role in the rapid responses to environmental stimuli and stress [23,24]. The GABAergic and the noradrenergic system are closely connected as demonstrated by reliable data in the literature showing GABA modulates the release of noradrenaline in the CNS. In particular, the GABAergic modulation involves presynaptic release-regulating GABAA receptors controlling noradrenaline exocytosis from noradrenergic nerve terminals [25,26]. Accordingly, the interplay between GABA and noradrenaline neurotransmission could explain the efficacy of pharmacological and/or nutraceutical approaches against mood illnesses. The hypothesis that TTBEs can influence this pathway also fully explains their effects when administered in vivo.

Based on these observations, we decided to investigate whether dietary supplementation with TTBEs, marketed for human consumption, modify motor and behavioral skills in an animal model. Our results support the idea that dietary integration with TTBEs represent a nutritional strategy to counteract stress and anxiety symptoms also in human.

2. Materials and Methods

2.1. Raw Samples

Tilia tomentosa Moench (Malvaceae) leaves were collected at embryonic stage as meristematic tissues: buds and young sprouts. The raw material was collected in the period February–April 2017 from plants spontaneously grown in the valleys of Chisone, Pellice, Germanasca, Bronda, and Varaita (Turin, Italy) and authenticated by a botanist. The manufacturing of the corresponding herbal preparations, Glyceric Macerates (GM), was performed by the encoded traditional method during 2017 in an Italian food supplements company (GEAL PHARMA—Turin, Italy). In detail, GM were prepared according to the European Pharmacopoeia 8th edition (Pharmaciens 1965), following the GM procedure deriving from the French Pharmacopoeia and adapted by the food supplements company using a cold maceration of the fresh raw material in a solution of water, 95% ethanol, and glycerol (50/20/30 *w*/*w*) with a 1:15 weight ratio between plant and solvent. The cold maceration process was protracted for 3 months, followed by a first filtration and, after 2 days of decanting, a second filtration (Whatman paper filter, n° 1, Sigma Aldrich, Milan, Italy). The obtained extracts (TTBEs), which represent the commercial products, were stored in dark bottles at normal atmosphere (N.A.), at 4 °C and 95% relative humidity until commercialization/use.

2.2. Spectroscopic Analysis: UV-Visible Fingerprint

UV–Visible absorption spectra (200–900 nm) were recorded by a spectrophotometer Agilent Cary 100 (Varian Co., Santa Clara, CO, USA) with 0.5 nm resolution, using rectangular quartz cuvettes with 1 cm path length. In order to avoid signal saturation GMs, before the spectroscopic analysis, were suitably diluted in the maceration solvent and thereafter spectra were acquired in duplicate and then averaged. The collection was performed at room temperature (25 ± 1 °C), against a blank solution represented by the dilution solvent. Standard normal variate (SNV) transform was later performed on the spectral data to remove or at least minimize any unwanted spectral contribution arising from multiplicative effects of scattering [27].

2.3. HPLC Analysis

In this study, fingerprint analysis for phytochemical characterization of samples were performed by different HPLC–DAD methods. Four polyphenolic classes were considered: benzoic acids (ellagic and gallic acids), catechins ((+)catechin and (−)epicatechin), cinnamic acids (caffeic, chlorogenic, coumaric, and ferulic acids), and flavonols (hyperoside, isoquercitrin, quercetin, quercitrin, and rutin). Total bioactive compound content (TBCC) was evaluated as the sum of the selected bioactive [27] compounds with health-promoting effects on human organism ("multimarker approach") [28]. Biomarkers were selected for their demonstrated positive healthy properties and antioxidant capacity by literature in relation to the use of this plant-derived products.

The chromatographic analysis was performed by an Agilent 1200 High-Performance Liquid Chromatograph equipped with an Agilent UV-Vis diode array detector (Agilent Technologies, Santa Clara, CA, USA). Bioactive molecules were separated on a Kinetex C18 column (4.6 × 150 mm, 5 m, Phenomenex, Torrance, CA, USA). Several mobile phases were analyzed and UV spectra were recorded at different wavelengths, according to [10,18], with minor modifications: (i) a solution of 10 mM KH_2PO_4/H_3PO_4 (A) and acetonitrile (B) with a flow rate of 1.5 mL·min^{-1} (method A—analysis of cinnamic acids and flavonols, gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min + 2 min of conditioning time); (ii) a solution (A) of methanol/water/formic acid (5:95:0.1 *v*/*v*/*v*) and a mix (B) of methanol/formic acid (100:0.1 *v*/*v*) with a flow rate of 0.6 mL·min^{-1} (method B—analysis of benzoic acids and catechins, gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min + 2 min of conditioning time). UV spectra were recorded at 330 nm (A); 280 nm (B).

All single compounds were identified in samples by comparison and combination of their retention times and UV spectra with those of authentic standards in the same chromatographic conditions.

2.4. Animals

Male and female mice (strain C57BL/6J) were purchased from Charles River (Calco, Italy). Mice were reared up to 3 and 24 months in the animal facility of the Department of Pharmacy, Section of Pharmacology and Toxicology, School of Medical and Pharmaceutical Sciences, University of Genoa (authorization n. 484 of 8th June 2004). The experimental procedures have complied the European legislation (Directive 2010/63/EU for animal experiments), the ARRIVE guidelines, and they were approved by the Committee on the Ethics of Animal Experiments of the University of Genoa and by the Italian Ministry of Health (DDL 26/2014 and previous legislation; permit number 50/2011-B and number 612/2015-PR).

2.5. Dietary Supplementation and Testing Procedure

Mice were assigned to the following groups for each condition (young male, old male, young female, and old female): water (w), ethanol and glycerol (vehi), and Tilia (TTBEs). All the treatments were performed orally; vehi and TTBEs were dissolved in the drinking water (500 µL in 500 mL). We decided the route and timing of treatments to limit the stress of animals according to the nutraceutical paradigms.

Animals were checked daily for the drugs intake and the weight was controlled before (day 0) and at the end (day 21) of the chronic administration.

2.6. Hole Board

The hole-board apparatus consists of black panel (40 × 40 cm, 2.2 cm thick) with 16 equidistant holes 3 cm in diameter in the floor. The board was positioned 15 cm above the table and divided into 9 squares of 10 × 10 cm. Each animal was placed singly in the center of the board and its behavior recorded with a video camera for 5 min. Head dippings, % of area explored, and the entries into the center were recorded according to [29]. Results are reported as means ± SEM.

2.7. Light/Dark Box

The light-dark box consists of two communicating sections one illuminated and the other dark (each comprising 35 × 30 × 21 cm). Each mouse was placed in the center of the light zone, and then the operator started to record 5 min of spontaneous exploration. Video recordings of mice behaviors were analyzed through the Tox Track software v2.83 [30].

2.8. Release Studies

Mice were sacrificed by cervical dislocation and promptly decapitated to collect the cortices. Each cortex was homogenized in 10 volumes of 0.32 M sucrose, buffered to pH 7.4 with Tris-(hydroxymethyl)-amino methane (Tris, final concentration (f.c.) 0.01 M) to prepare purified synaptosomes [31]. The homogenate was centrifuged at 1000× *g* for 5 min and the supernatant was stratified on a discontinuous Percoll gradient (2%, 6%, 10%, and 20% *v*/*v* in Tris-buffered sucrose) and centrifuged at 33,500× *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellets were resuspended in a physiological solution with the following composition (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; $CaCl_2$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; HEPES, 10; glucose, 10; pH 7.2–7.4.

2.9. Experiments of Release

Purified nerve endings were incubated for 15 min a 37 °C with [3H]noradrenaline ([3H]NA; f.c.: 30 nM) in the presence of 0.1 μM 6-nitroquipazine and 0.1 μM GBR12909 to block false labelling of serotonergic and dopaminergic synaptosomes, respectively. An equal amount of synaptosomal suspension was then stratified on microporous filters at the bottom of parallel chambers in a Superfusion System (Ugo Basile, Comerio, Varese, Italy) [32] and maintained at 37 °C. Isolated nerve endings were superfused with the physiological solution for 38 min to balance the system and then exposed to muscimol. Eight minutes before agonist the synaptosomes were exposed to TTBEs or antagonists. Fractions and filters were collected as above and measured for radioactivity according to [33].

2.10. Statistical Analysis

The univariate statistical analysis and the correspondent graphical representation were carried out by using Past 3 or 4 [34]. An analysis of variance was performed by ANOVA followed by Tukey's multiple comparison test. Data are presented as the mean ± standard error of the mean (SEM) and considered significant for $p < 0.05$ at least. Multivariate data analysis has been performed by CAT (Chemometric Agile Tool) software (August 27, 2020), one advanced chemometric multivariate analysis tool based on R, developed by the Chemistry Group of the Italian Chemical Society (http://gruppochemiometria.it/index.php/software). PCA was applied as explorative multivariate statistical method of unsupervised pattern recognition to rationalize the useful information of a data set [35,36]. Each column (variable) of the data set (data matrix) under study is considered as an axis in the multi-dimensional space and each row (object) of the data set under study is a point in this space.

PCA algorithm searches for the maximum variance direction, the first principal component (PC1), corresponding to a high amount of information in the multidimensional space of the original data. The second principal component (PC2) is the direction having the largest remaining variance among all directions orthogonal to the first PC. As well as each succeeding component has as much of the remaining variability as possible which is not explained by the previous PCs. In this way, the large amounts of complex information, the multivariate original data matrix, is rationalized by way of simple bidimensional or tridimensional plots. Few animals were not included in the data when they showed lesions or complete immobility (*). Moreover, some (<3%) of video examinations were not included in the data analysis due to the video quality or software limits.

2.11. Drugs

1-[7,8 3H]-noradrenaline (specific activity 39 Ci $mmol^{-1}$) was from Perkin Elmer. TTBEs from Gealpharma and muscimol bicucullin and all standards for HPLC analysis were from Sigma Aldrich (Milan, Italy). 6-Nitroquipazine maleate was donated from Duphar, Amsterdam, The Netherlands. 1-(2-(Bis-(4-fluorophenyl) methoxy) ethyl)-4-(3-phenylpropyl) piperazine dihydrochloride (GBR12909) was purchased from Tocris Bioscience (Bristol, UK). Ethanol was supplied by VWR International S.r.l (Milan, Italy).

3. Results

3.1. Bud-Extracts Characterization: UV-Visible and HPLC Fingerprints

The quality control of the commercial bud-derivatives has been performed using both an untargeted spectroscopic fingerprint and a targeted chromatographic fingerprint as previously reported by the authors [37–41]. As far as the first one is concerned, UV-Visible spectroscopy was employed in a screening step in order to obtain a rapid preliminary untargeted fingerprint of the extract (after Standard Normal Variate—SNV pre-treatment) and followed by the targeted fingerprint by HPLC chromatography. Phytochemical fingerprint showed that benzoic acids (560.61 ± 31.92 mg/100 g FW) and catechins (424.20 ± 41.41 mg/100 g FW) represented the main phenolic classes in the analysed bud-extracts (47% and 36% of the total phytocomplex, respectively), followed by flavonols (166.34 ± 14.02 mg/100 g FW, 14% of the total phytocomplex) and cinnamic acids (32.02 ± 2.80 mg/100 g FW, 3% of the total phytocomplex) as reported in Table 1.

Table 1. Main phytochemicals in *T. tomentosa* bud-extract.

Chemical Class	Phytochemical	mg/100 g of Fresh Weight (FW)
Cinnamic acids	Caffeic acid	n.d.
	Chlorogenic acid	n.d.
	Coumaric acid	n.d.
	Ferulic acid	32.02 ± 2.80
Flavonols	Hyperoside	32.98 ± 3.46
	Isoquercitrin	10.45 ± 3.67
	Quercetin	116.75 ± 4.95
	Quercitrin	4.38 ± 1.59
	Rutin	1.78 ± 0.36
Benzoic acids	Ellagic acid	440.21 ± 21.02
	Gallic acid	120.40 ± 10.90
Catechins	(+)-Catechin	203.56 ± 31.39
	(−)-Epicatechin	220.64 ± 10.03

The results are expressed as mean ± S.D. n.d. = not detected.

In Figure 1, UV-Visible spectra, HPLC fingerprint, and bioactive compound quantification of the *T. tomentosa* bud-extract were reported.

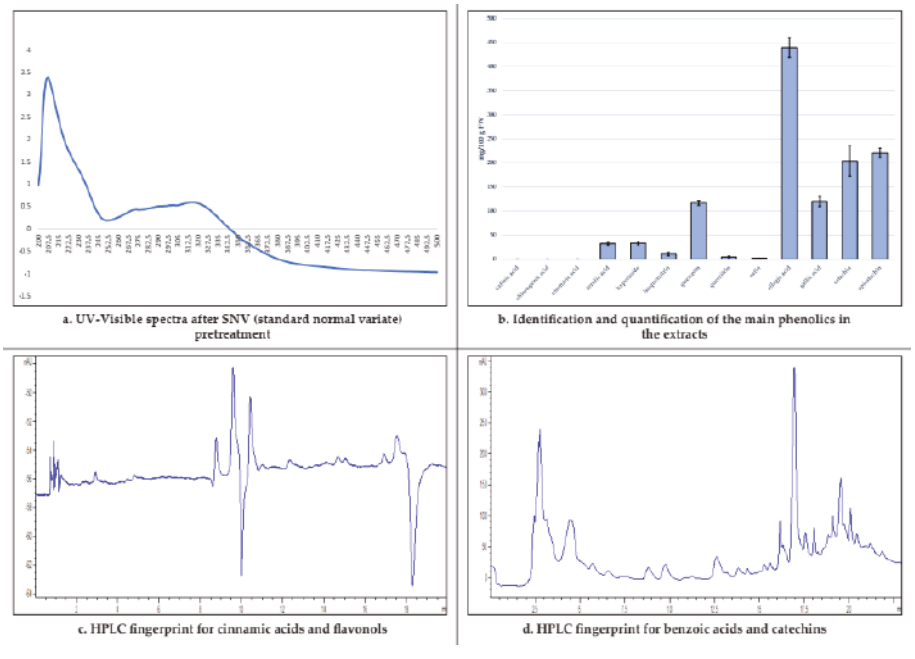


Figure 1. UV-Vis spectra (**a**), bioactive compound quantification (**b**), and HPLC fingerprint (**c**,**d**) of the *T. tomentosa* bud-extracts.

3.2. Evaluation Active Dilutions of TTBEs on Native Brain Targets

Cortical noradrenergic synaptosomes express functional GABAA receptors modulating noradrenaline exocytosis as previously demonstrated in rats by Schmid and colleagues in 1999. Accordingly, in our experimental conditions, muscimol induces a concentration-dependent stimulation of these receptors able, in cascade, to elicit noradrenaline release in a bicucullin-sensitive manner (1 μM; Figure 2a). Data in the literature, published adopting an electrophysiological approach, suggest that TTBEs mimic GABA and benzodiazepines at GABAA receptors [20]. Consequently, we selected a submaximal concentration of muscimol to investigate the modulatory role of TTBEs on presynaptic GABAA receptors regulating noradrenaline release. Progressive water-dilutions of commercial bud extracts are utilized during in vitro superfusion experiments from cortical synaptosomes. Figure 2b demonstrates that TTBEs potentiated the 10 μM muscimol induced noradrenaline release in a concentration-dependent manner. Interestingly, 1:2000 TTBEs provoked a significant increase in the muscimol induced [3H] noradrenaline release that is also confirmed at lower dilutions. Interestingly, 1:2000 TTBEs was ineffective on noradrenaline basal release (data not shown).

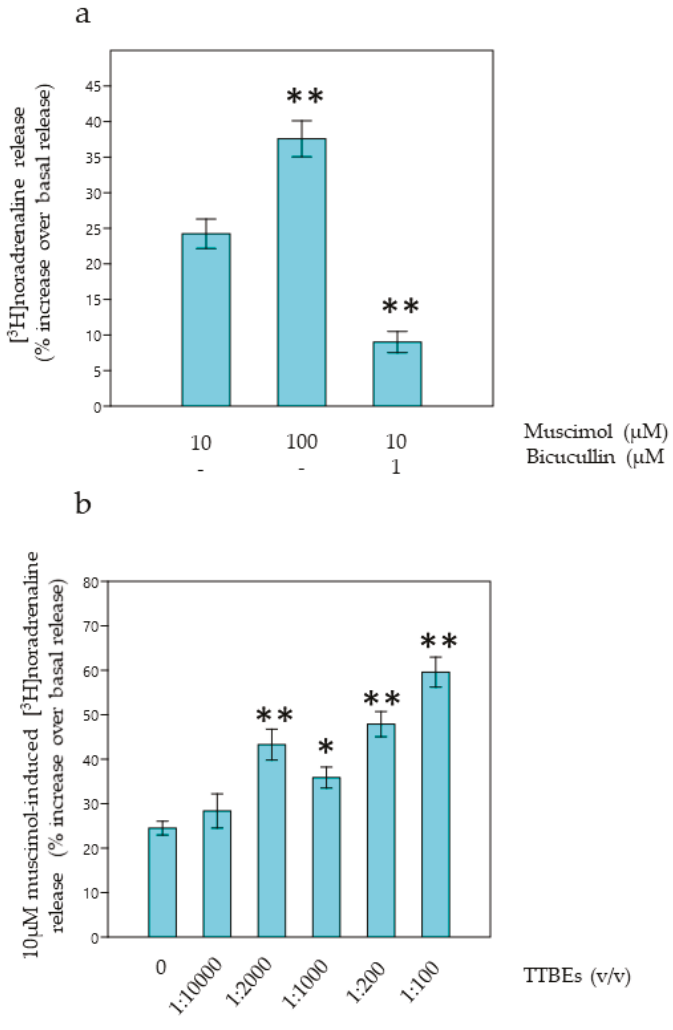


Figure 2. In vitro noradrenaline release from cortical synaptosomes of young male mice: functional activity of native GABAA receptors in presence of TTBEs dilutions. (**a**) Counteracting effect of 1 μM bicucullin on muscimol evoked [3H]noradrenaline release (**b**). Effects of TTBEs on 10 μM muscimol evoked [3H]noradrenaline release. Data represent the mean ± S.E.M. from six young male mice. Statistical analysis was performed by applying ANOVA followed by the Tukey's Multiple Comparison test. * $p < 0.05$, ** $p < 0.01$ vs. 10 μM muscimol.

3.3. Dietary Supplementation with TTBEs: Control of Daily Intake and Weight Gain

Basing on in vitro results, we start with the in vivo behavioural evaluations of anxiolytic TTBEs properties. A total of 21 days of chronic oral administration of 1:2000 TTBEs in drinking water involved 4 animal groups divided according to gender and age profile (young male, young female, old male, and old female). Moreover, each group is separated into three subgroups based on the drinking solutions (water, vehicle, and TTBEs) to accurately discriminate possible effects due to the presence of ethanol and glycerin. During the oral supplementation, animals are monitored to evaluate changes in water consumption and body weight. Data displayed in

Table 2 demonstrated that neither the vehicle nor the TTBEs significantly modify the amount of liquid drunk. Accordingly, in each group, the weight growth was significantly unaltered by vehicle or TTBEs.

Table 2. Weight gain and water consumption.

Animals	Treatment	Water Consumption	Weight			*n*
			Start	End	Variation	
YM	W	6.38 ± 0.63	26.4 ± 0.3	27.1 ± 0.3	(+2.8%)	15
	Vehi	6.12 ± 0.55	27.9 ± 0.5	29 ± 0.6	(+4.3%)	26
	TTBEs	6.91 ± 0.26	27 ± 0.2	28.3 ± 0.3	(+5%)	24
YF	W	5.94 ± 0.45	22 ± 0.2	22.5 ± 0.3	(+2.3%)	14
	Vehi	5.67 ± 0.38	21.7 ± 0.2	22.6 ± 0.1	(+4.1%)	20
	TTBEs	5.39 ± 0.58	20.5 ± 0.1	20.9 ± 0.1	(+2.2%)	26
OM	W	6.65 ± 0.13	29.2 ± 0.5	29.2 ± 0.4	(−0.1%)	12
	Vehi	6.12 ± 0.48	30.6 ± 0.5	30.5 ± 0.5	(−0.2%)	12
	TTBEs	6.78 ± 0.19	33.6 ± 0.4	33.7 ± 0.3	(+0.3%)	12
OF	W	4.91 ± 0.62	25.6 ± 0.5	25.8 ± 0.5	(+0.8%)	14
	Vehi	4.37 ± 0.52	26.5 ± 0.3	26.2 ± 0.2	(−1.4%)	18
	TTBEs	3.83 ± 0.33	26.7 ± 0.3	26.3 ± 0.4	(−1.7%)	18

The results are expressed as mean ± S.E.M. YM: young male, YF: young female, OM: old male, OF: old female, W: water, Vehi: vehicle, TTBEs: *Tilia tomentosa* Moench Buds Extracts.

3.4. Dietary Supplementation with TTBEs: Behavioral Scores

Animal behavioral performances in the hole board test (n° head dippings—%HD, % of area explored—%AE, % of entries into center—%C) and in light dark box test (time in light—tL, n° of transitions—T, average speed—Av Sp, exploration rate %—RAE and total distance—D) are analyzed before (day 0) and at the end (day 21) of the dietary supplementation.

The corresponding data matrix $D_{16,8}$ has been reported in Table 3.

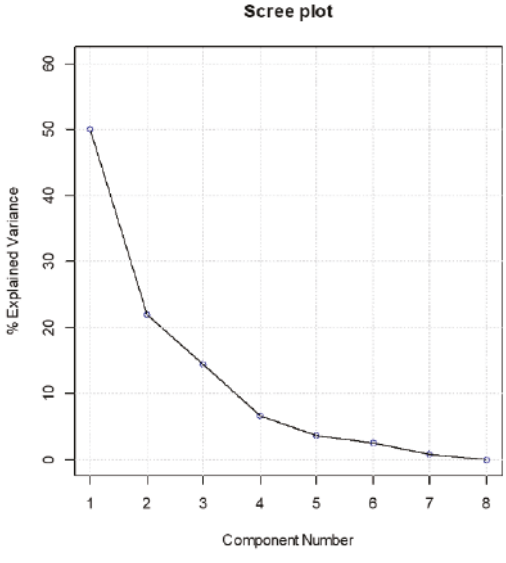
Table 3. Data matrix $D_{16,8}$: 16 animal groups, classified both by age (c1) and gender (c2), and the 8 behavioral scores.

	c1	c2	%HD	%AE	%C	tL	T	Av Sp	RAE	D
YMW	Y	M	4.50	38.89	22.92	36.00	6.25	7.62	87.60	2375.00
YMVehi	Y	M	12.00	59.26	24.37	46.00	7.33	7.52	76.67	2350.00
YMTTBEs	Y	M	17.22	83.95	18.88	56.00	10.45	11.55	84.42	3586.00
YFW	Y	F	5.83	50.00	10.28	21.25	3.75	9.34	79.50	2918.00
YFVehi	Y	F	9.12	63.89	6.59	60.11	6.78	9.88	78.83	3074.00
YFTTBEs	Y	F	19.75	69.44	5.42	50.82	9.00	6.15	70.60	1920.00
OMW	O	M	2.33	22.22	33.33	71.00	5.50	4.10	45.20	1124.00
OMVehi	O	M	3.17	38.89	47.22	51.50	4.67	4.05	48.67	1282.00
OMTTBEs	O	M	11.29	66.67	25.01	59.00	4.25	6.38	43.00	2104.00
OFW	O	F	7.67	55.00	13.42	52.50	7.67	4.21	48.00	1224.00
OFVehi	O	F	13.80	75.55	9.51	30.00	3.33	4.31	47.00	1358.00
OFTTBEs	O	F	11.17	70.37	7.26	35.16	3.60	4.70	46.00	1527.00
ZeroYMW	Y	M	30.41	94.95	5.73	58.69	8.23	12.54	46.75	3911.75
ZeroYFW	Y	F	31.42	93.98	8.15	71.38	8.00	11.24	60.50	3508.50
ZeroOMW	O	M	28.35	93.46	12.63	95.67	6.22	6.97	55.25	2210.00
ZeroOFW	O	F	27.00	82.64	13.87	58.31	7.07	7.19	50.67	2309.17

YMW: young male water, YFW: young female water, YMVehi: young male vehicle, YFVehi: young female vehicle, YMTTBEs: young male *Tilia tomentosa* Moench Buds Extracts, YFTTBEs: young female *Tilia tomentosa* Moench Buds Extracts, ZeroYMW: day 0 young male water, ZeroYFW: day 0 young female water, OMW: old male water, OFW: old female water, OMVehi: old male vehicle, OFVehi: old female vehicle, OMTTBEs: old male *Tilia tomentosa* Moench Buds Extracts, OFTTBEs: old female *Tilia tomentosa* Moench Buds Extracts, ZeroOMW: day 0 old male water, ZeroOFW: day 0 old female water.

In details, the data matrix $D_{16,8}$, whose rows are the objects (the first 12 correspond to the treated animals and the last 4 to the animals at the zero conditions) and whose columns are the results of the 8 behavioral test investigated, was taken into account. The objects are described by 8 experimental variables and by 2 categories (c1: age and c2: sex). PCA was applied as unsupervised pattern recognition technique in order to explore the mice behavior information using a multivariate approach [35,36]. PCA was performed after the autoscaling pre-processing of the data matrix in order to elaborate the multivariate data characterized by different scales and units. This pre-processing technique commonly used in multivariate analysis, consists of mean-centering followed by the division of each column of the data matrix by its standard deviation [35].

The first 2 principal components (PC1-PC2) explained the 72% of the total variance/information of the data set as highlighted in Figure 3.



PC	Eigenvalue	% variance
1	4.00454	50.057
2	1.75982	21.998
3	1.15383	14.423
4	0.532143	6.6518
5	0.286999	3.5875
6	0.197506	2.4688
7	0.0643822	0.80478
8	0.000781768	0.0097721

Figure 3. Scree plot: % explained variance of each PCs.

Figure 4 shows the PCA score plot, biplot (scores plus loadings plot), and loading plots on the first to the second PCs obtained from the above-mentioned data matrix.

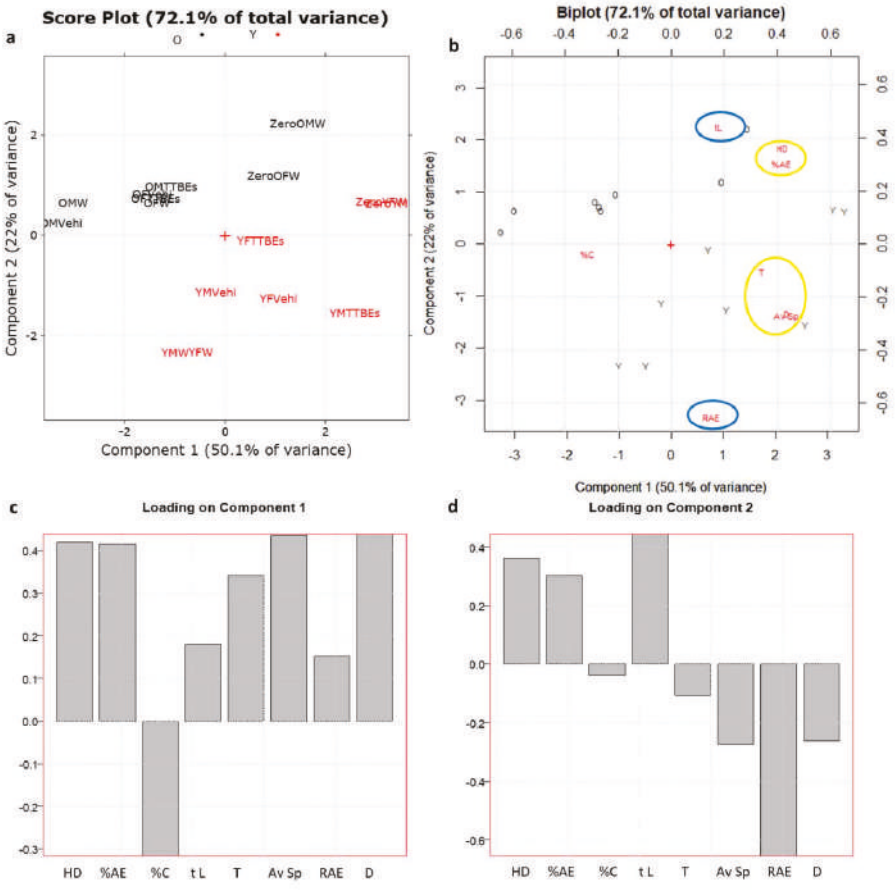


Figure 4. (**a**) PC1-PC2 score plot and biplot of the $D_{16,8}$ data matrix. (**b**) PC1-PC2 biplot (scores plus loadings plot). Animals are categorized by age: old mice are reported in black and young ones in red, respectively. (**c**) Loading plot on PC1. (**d**) Loading plot on PC2. YMW: young male water, YFW: young female water, YMVehi: young male vehicle, YFVehi: young female vehicle, YMTTBEs: young male *Tilia tomentosa* Moench Buds Extracts, YFTTBEs: young female *Tilia tomentosa* Moench Buds Extracts, ZeroYMW: day 0 young male water, ZeroYFW: day 0 young female water, OMW: old male water, OFW: old female water, OMVehi: old male vehicle, OFVehi: old female vehicle, OMTTBEs: old male *Tilia tomentosa* Moench Buds Extracts, OFTTBEs: old female *Tilia tomentosa* Moench Buds Extracts, ZeroOMW: day 0 old male water, ZeroOFW: day 0 old female water, HD: n° head dippings, %AE: % of area explored, %C: % of entries into center, tL: time in light, T: n° of transitions, Av Sp: average speed, RAE: exploration rate %, D: total distance.

PC1, the direction of maximum variance which explains the 50.1% of the total information, allows a good discrimination between the objects according to the different treatments (W: water; Vehi: vehicle; TTBES: Tilia). Particularly, as shown in Figure 4a, TTBEs move objects to the animals at the starting conditions (indicated as zero) which have higher scores on PC1. Regarding young animals (in red), these zero conditions are the same both for male and for female, while in the case of old mice (in black) are different between the genders.

On PC2, which explains the 22% of the remaining variance, the old animals (in black) are separated from the young ones (in red). As showed in Figure 4b,c, the HD (n° of head dippings), the %AE

(% of area explored), D (total distance) and Av Sp (average speed), highlighted in yellow in the biplot, had highest loadings on PC1. Instead, variables obtained from the light dark box test such as the time in light (tL) and the exploration rate (RAE), highlighted in blue in the biplot, had highest loadings on PC2 and they resulted important to separate young and old animals (Figure 4d). The PCA analysis of our data clearly demonstrated that young and old animals are very discernible by the variable included. Indeed, the age-dependent behaviors are strongly described by the PC2 that is positively associated with tL and RAE. According to previous results, repeated exposure to a novel apparatus produced a reduction in exploration parameters due to a process commonly referred to as habituation [42]. Indeed, in our experimental conditions, most of the behavioral parameters were decreased in water groups compared with the scores in time 0 section (Table 3). Curiously, the habituation seemed more effective in the young male group and less potent in the old female group. Across the age and gender groups, this phenomenon appeared less active on two parameters (%C and RAE). To precisely evaluate the effects of the oral supplementation and the gender dependency, we decided to normalize each animal score, subtracting the respective time 0 section scores. In detail, two separate matrices were created ($M_{6,8}$ for male mice and $F_{6,8}$ for female ones, respectively) by subtracting the corresponding zero condition for each object (Figure 5).

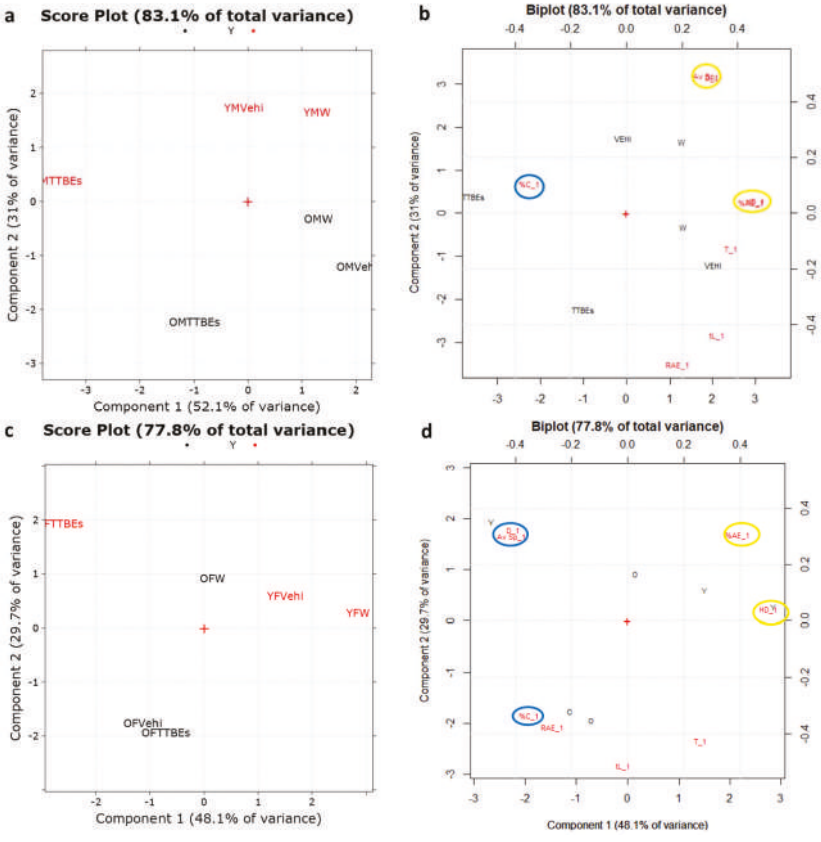


Figure 5. PC1-PC2 score plot (**a**) and biplot (**b**) of the $M_{16,8}$ data matrix. PC1-PC2 score plot (**c**) and biplot (**d**) of the $F_{16,8}$ data matrix. tL_1: time in light day 21-day 0, HD_1: head dippings day 21-day 0, AE_1: % of area explored day 21-day 0, RAE_1: exploration rate % day 21-day 0, %C_1: % of entries into center day 21-day 0, Av Sp_1: average speed day21-day0, T_1: n° of transitions day 21-day 0, D_1: distance day 21-day 0, W: water, Vehi: vehicle, TTBEs: *Tilia tomentosa* Moench Buds Extracts.

Figure 5a shows the PC1-PC2 score plot of $M_{6,8}$ data matrix corresponding to the male animals, which explains the 83.1% of the total variance/information of the data set. In this plot, the effects of TTBEs treatment on young (in red) and old (in black) mice, respectively, are better highlighted. In fact, both for young and old mice, the TTBEs animals separate on PC1 from the corresponding ones treated with W or Vehi. On PC2, as previously already described, old animals (in black, higher scores on PC2) are separated from the young ones (in red, lower scores on PC2). After the TTBEs treatment the HD, % AE, Av Sp, and D variables increase (Figure 5b) and consequently the differences with respect to the zero conditions decrease, i.e., HD_1, % AE_1, Av Sp_1, and D_1 (highlighted in yellow). On the contrary as concerns %C variable, the difference with respect to the zero conditions (i.e., %C_1 highlighted in blue) increases.

As regards young females (in red), the PC1-PC2 score plot of $F_{6,8}$, which almost explains the 78% of the total variance, shows a similar trend to male mice (Figure 5c). Particularly after the TTBEs treatment, the HD and % AE increase (Figure 5d, loadings highlighted in yellow) while % C, Av Sp, and D decrease determining an increase in differences compared to the corresponding zero condition (i.e., % C_1, Av Sp_1, D_1: in blue). The two obtained distinct matrices for male and female animals are reported in Appendix A.

Then we performed a post-hoc analysis on each behavioural variable to better characterize the anxiolytic effect of TTBEs. It has been previously reported that the exposure of animals to various stressful stimuli decreases some exploratory behaviours [43,44]. In the hole-board test, a pronounced inhibition of head-dipping behaviour was observed in animals that had been exposed to stressful stimuli [45]. The results with the univariate statistical analysis demonstrated a significant increase in the number of head dippings in young animals and old male mice after 21 days of TTBEs treatment (Figure 6a). Moreover, the chronic oral treatment with bud extracts increased the total amount of locomotion, expressed as % of area explored (Figure 6b), both in young and in old male mice. Although the TTBEs did not increase the %center entries (Figure 6c), both curiosity and exploration were modified which is consistent with a reduction in anxiety-like behaviour. Conversely, the HB scores in females are insensitive to dietary integration with the exception of HD in younger animals (Figure 7a).

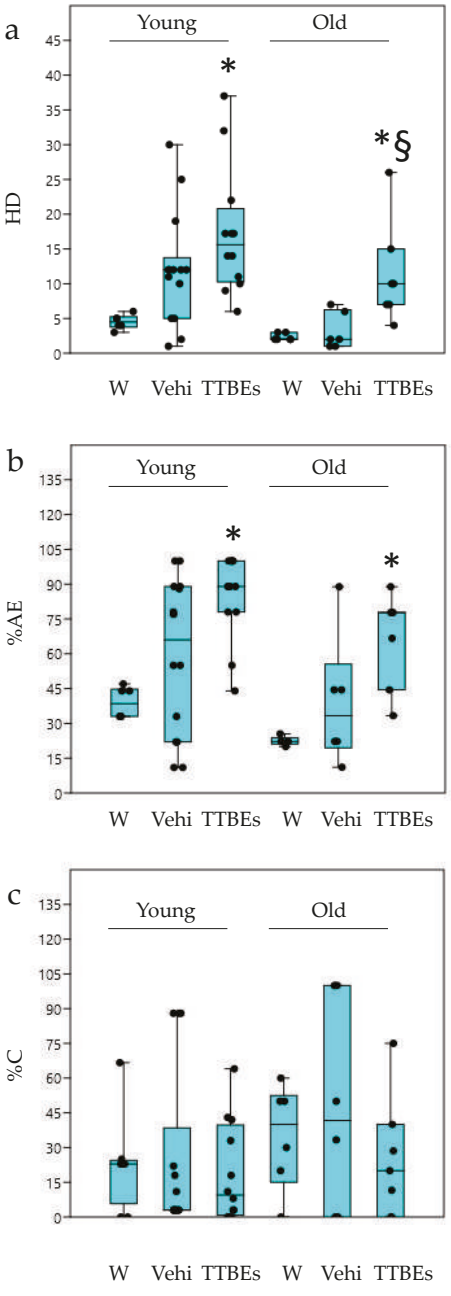


Figure 6. Hole Board test in young and old male mice: effects 21 days of dietary integration with vehicle, TTBEs respect to water. Behavioral skills were quantified as (**a**) n° head dippings (%HD), (**b**) % of area explored (%AE), (**c**) % of entries into center (%C). Data represent the mean ± S.E.M. (n = 6 (W), 14 (Vehi) 12 (TTTBEs) young and (n = 6 (W), 6 (Vehi) 6 (TTTBEs) old mice analyzed in three different trials. Statistical analysis was performed by applying ANOVA followed by the Tukey's multiple comparison test. * $p < 0.05$; $p < 0.01$ vs. water; § $p < 0.05$ vs. vehicle.

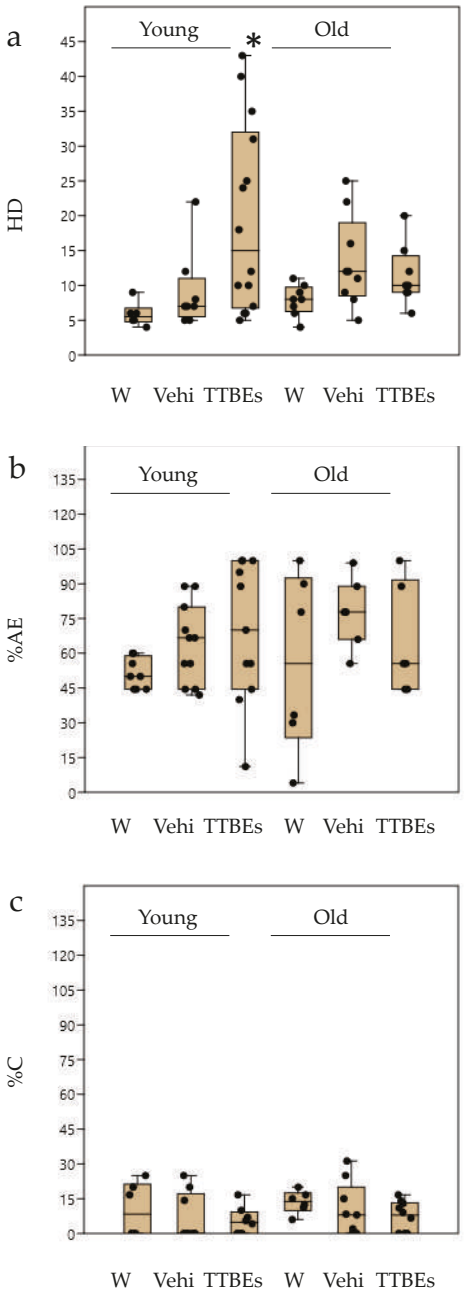


Figure 7. Hole Board test in young and old female mice: effects 21 days of dietary integration with vehicle, TTBEs respect to water. Behavioral skills were quantified as (**a**) n° head dippings (%HD), (**b**) % of area explored (%AE), (**c**) % of entries into center (%C). Data represent the mean ± S.E.M. (n = 6 (W), 11 (Vehi) 14 (TTTBEs) young and (n = 8 (W), 9 (Vehi) 9 (TTTBEs) old mice analyzed in three different trials. Statistical analysis was performed by applying ANOVA followed by the Tukey's multiple comparison test. * $p < 0.05$ water.

In the LD paradigm, TTBEs dietary supplementation produced a significant increase in transitions in young male mice coupled with a positive trend in the speed and distance scores (Figure 8). Conversely, the oral administration of Tilia extracts did not significantly modify the LD score in female mice (Figure 9). Interestingly, some of the LD parameters appeared modified by the consumption of vehicle, confirming a susceptibility of females to ethanol.

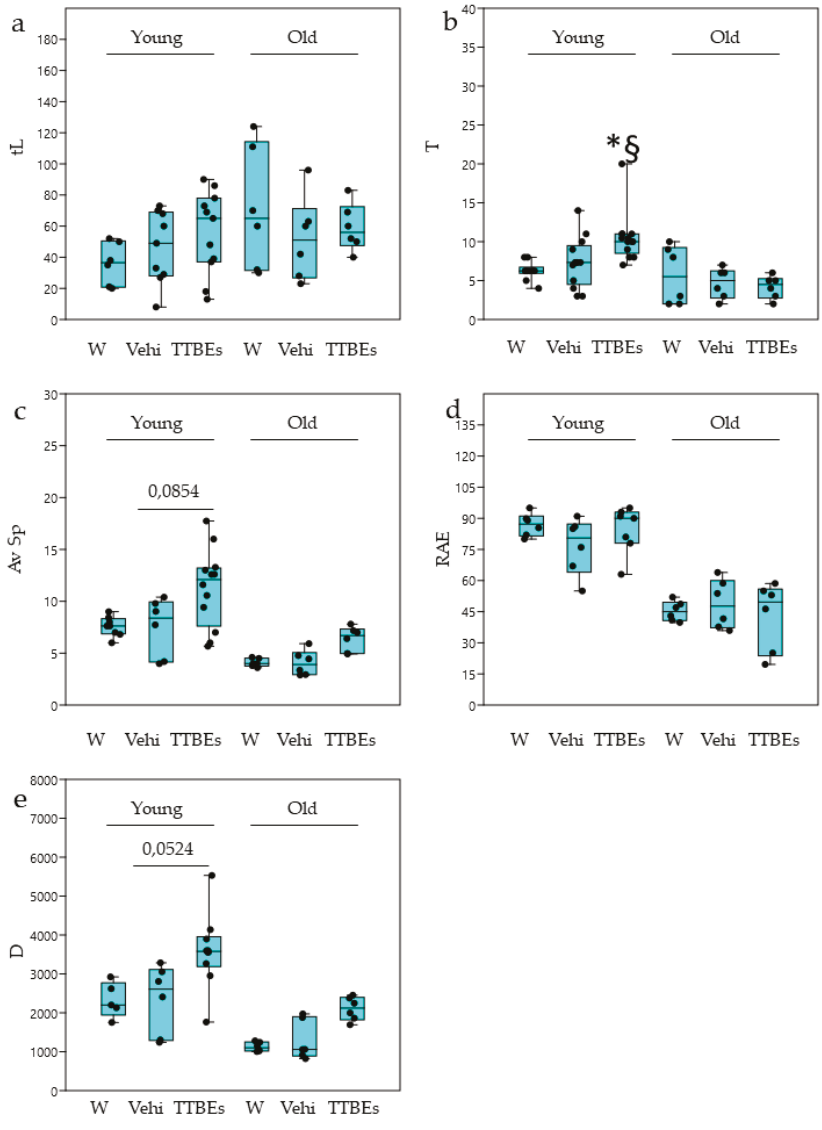


Figure 8. Light Dark test in young and old male mice: effects 21 days of dietary integration with vehicle, TTBEs respect to water. Behavioral skills were quantified as (**a**) time in light (tL), (**b**) n° of transitions (T), (**c**) average speed (Av Sp), (**d**) exploration rate % (RAE) and (**e**) total distance (D). Data represent the mean ± S.E.M. (n = 9 (W), 12(Vehi) 12 (TTTBEs) young and (n = 6 (W), 6 (Vehi) 6 (TTTBEs) old mice analyzed in three different trials. Statistical analysis was performed by applying ANOVA followed by the Tukey's multiple comparison test. * $p < 0.05$ water; § $p < 0.05$ vs. vehicle.

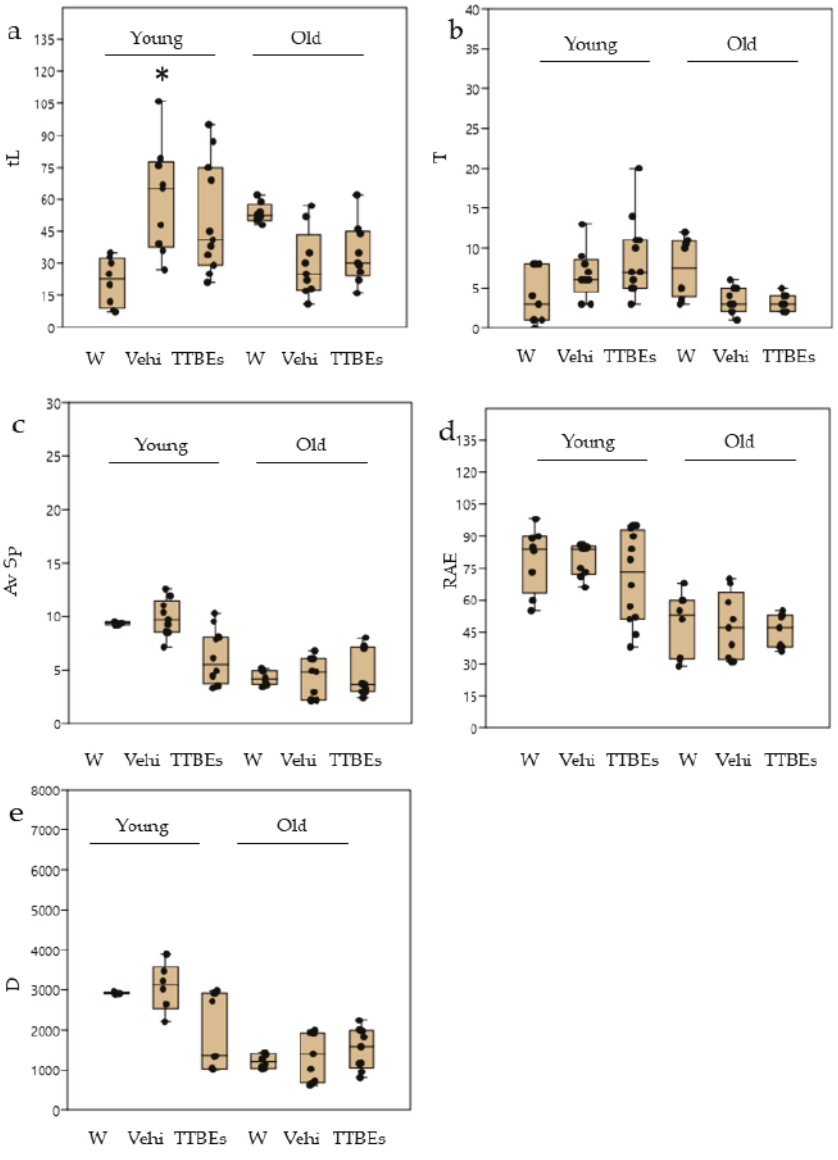


Figure 9. Light Dark test in young and old female mice: effects 21 days of dietary integration with vehicle, TTBEs respect to water. Behavioral skills were quantified as (**a**) time in light (tL), (**b**) n° of transitions (T), (**c**) average speed (Av Sp), (**d**) exploration rate % (RAE) and (**e**) total distance (D). Data represent the mean ± S.E.M. (n = 8 (W), 9 (Vehi) 12 (TTTBEs) young and (n = 6 (W), 9 (Vehi) 9 (TTTBEs) old mice analyzed in three different trials. Statistical analysis was performed by applying ANOVA followed by the Tukey's multiple comparison test. * $p < 0.05$ water.

4. Discussion

Accumulating evidence from animal and human research reinforce the concept of the microbiome–gut–brain axis. Indeed, microbiome regulates acknowledged functions of the CNS, the immunity system and behavior in health and disease. Moreover, the diet may modulate gut

microbiome, altering the nutrient availability [46]. Interestingly, anxiety disorders are common and complex psychiatric syndromes susceptible to the influence of microbiome signaling [47,48]. This aspect is extremely important considering the global number of people affected by stress-related disorders. Curiously, some categories seem to be more susceptible as documented by the literature showing that aging typically produces an increase in anxiety vulnerability and sedative consumption [49–52]. Furthermore, gender vulnerability seems to emerge in a context of great complexity [53,54]. If this is not enough, the approved pharmacological treatments cause severe side effects like hallucinations, addiction, suicide, insomnia, and loss of motor coordination. Therefore, alternative strategies that combine different approaches are fundamental in line to resolve some of these peculiarities. Dietary integration with nutraceutical supplements could represent an intriguing plan for improving the efficacy and the safety of synthetic anxiolytics. At this regard, we decided to investigate an in vivo oral supplementary protocol with commercial buds extracts of *Tilia Tomentosa* in rodents combining the analysis of different behavioral parameters. In details, we studied the role of aging, gender, and nutrients, alone or in combination, on the stress-related response of C57BJ mice. Our results demonstrated that 21 days of dietary integration with TTBEs produce anxiolytic effects in mice. This evidence is in accordance with previous in vitro results describing GABA and benzodiazepine-like actions evoked by TTBEs [20]. Moreover, our research confirms the anxiolytic properties of active compounds present in the biological matrix of *Tilia Tomentosa* [19,55–59]. To be specific, results presented here demonstrate that active nutrients, extracted from buds and freely administered through the drunk water, can modify mice behavior with gender and age specificity. Interestingly, our monitoring of liquid consumption per day revealed that dietary integration with TTBEs are well accepted and all the animals do not change the hydration rate. Accordingly, the different mice groups displayed a weight trend in line with the respective water groups. Combined statistical analysis revealed that male mice seemed more sensitive to the natural supplements modifying both motor and curiosity score. Interestingly, young mice demonstrated the maximum performance increase both in HB and LD test. Old male mice reached a significant increase in the number of HD and the %AE but fail to ameliorate the LD test parameters. These results need further investigations focused, among other things, on the diverse composition of the gut microbiome [60–62] and the diverse sensitivity for light in ancient mice [63]. On the other hand, we cannot exclude the possibility that increasing concentrations of TTBEs or a longer time of supplementation can be effective. However, in line with the nutraceutical standpoint, we decided to administrate TTBEs at the maximum dilution rate according to our in vitro results on native brain targets. Accordingly, we do not have performed the gavage to avoid additional stress stimuli. Interestingly, the analysis of female trends reveals a different sensitivity to TTBEs. Old females seem to be completely unaffected by dietary supplementation. This evidence reinforces the idea, based on male results, that aging modifies animal sensitivity to TTBEs. Likewise, young female reveals low sensitivity to supplements, showing only a positive trend in the HB results. We are aware that results from rodents may not be equivalent to those in human. Although, TTBEs are commercial products commonly integrated in the human diet according to the paradigms of traditional medicine. The generalized lack of efficacy in female mice could be explained in part to the action of vehicle constituents. Indeed, water and vehicle groups are fundamental controls fixed in our protocol to elucidate the impact of glycerol and ethanol on mice. Ethanol is a potent modulator of GABAA receptors, and some studies have convincingly demonstrated the sedative effects both in male and female rodents [63–65]. The LD score of female groups describes a predictable modulation of behaviors mediated by vehicle. We observed a common trend for TTBEs and vehicle in two LD parameters (tL and T) and a different trend in (Av Sp and D). Interestingly, PCA analysis also indicated that vehicle closely resembles TTBEs in old females. Although not significative, these data support the idea that low doses of ethanol could modify the LD scores in females. Indeed, females possess enzymatic and hormonal characteristics enhancing ethanol influence [66–68]. Furthermore, estrogens also interfere with anxiety and motor performances [69–71]. Future research will be dedicated to correlate the female response to their hormonal profile.

On the other hand, we cannot exclude the possibility that bioactive compounds present in TTBEs produce diverse actions on the basis of the animal peculiarities. Furthermore, we must keep in mind that TTBEs are a mixture of different active compounds extracted from a vegetal matrix. The efficacy of these supplements is strictly linked to the presence of standard quality control. In this regard, we also describe a bioactive compounds quantification of TTBEs nutrients revealing a remarkable presence of catechins, benzoic acids, and flavonols. Interestingly, many of the substances detected, like quercetin, ellagic acid, gallic acid, and catechin, are also associated with anxiolytic effects in the literature [72–78] but a detailed assessment of the bioavailability of these compounds by gender and age in mice is still lacking.

5. Conclusions

In summary, dietary integration with TTBEs reduce anxiety-related behavior in mice showing different efficacy depending on the age and gender characteristics and without apparent side effects. Moreover, our data highlight the importance of limiting ethanol concentration waiting to determinate the precise mechanism of action. Overall taking into account the results, we described for the first time the efficacy of TTBEs after oral consumption. Finally, our results support the idea that an integration of human diet with botanicals could represent an improvement in the therapeutic protocols of anxiety disorders.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Data matrix $M_{6,8}$: male mice.

	c1	c2	HD_1	%AE_1	%C_1	tL_1	T_1	Av Sp_1	RAE_1	D_1
YMW	Y	M	25.90909	56.05949	−17.1947	22.69231	1.980769	4.915	−40.85	1536.75
YMVehi	Y	M	18.40909	35.68949	−18.6447	12.69231	0.900769	5.015	−29.92	1561.75
YMTTBEs	Y	M	13.18909	10.99949	−13.1547	2.692308	−2.21923	0.985	−37.67	325.75
OMW	O	M	26.02294	71.24405	−20.6994	24.66667	0.72	2.865	10.05	1086
OMVehi	O	M	25.18294	54.57405	−34.5894	44.16667	1.55	2.915	6.58	928
OMTTBEs	O	M	17.06294	26.79405	−12.3794	36.66667	1.97	0.585	12.25	106

Table A2. Data matrix $F_{6,8}$: female mice.

	c1	c2	HD_1	%AE_1	%C_1	tL_1	T_1	Av Sp_1	RAE_1	D_1
YFW	Y	F	25.58667	43.98148	−2.12525	50.125	4.25	1.895	−19	590.5
YFVehi	Y	F	22.29667	30.09148	1.564749	11.265	1.22	1.355	−18.33	434.5
YFTTBEs	Y	F	11.66667	24.54148	2.734749	20.555	−1	5.085	−10.1	1588.5
OFW	O	F	20.68294	38.46405	−0.78936	43.16667	−1.45	2.755	7.25	986
OFVehi	O	F	14.55294	17.91405	3.120638	65.66667	2.89	2.655	8.25	852
OFTTBEs	O	F	17.18294	23.09405	5.370638	60.50667	2.62	2.265	9.25	683

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Supplementation with Resveratrol, Piperine and Alpha-Tocopherol Decreases Chronic Inflammation in a Cluster of Older Adults with Metabolic Syndrome

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Abstract: Metabolic Syndrome (MetS) is increasing worldwide regardless of culture, genetic, gender, and geographic differences. While multiple individual risk factors, such as obesity, hypertension, diabetes, and hyperlipidemia, can cause cardiovascular disease (CVD), it is the intercurrence of these risk factors that defines MetS as a cluster that creates an environment for atherosclerosis and other manifestations of CVD. Despite the advances in the knowledge and management of each of the components of MetS, there are two molecular biology processes, chronic inflammation and oxidative stress, which are still underdiagnosed and undertreated. In order to assess the effect of a dietary supplement on chronic inflammation in MetS, we conducted a clinical trial with volunteers receiving a formula composed of resveratrol, piperine and alpha tocopherol (FRAMINTROL®), together with their habitual treatment, for three months. The inflammatory state was evaluated by ultrasensitive C reactive protein (US CRP) and ferritin in plasma, and oxygen consumption and chemiluminescence in neutrophils. The results showed that ferritin decreased by 10% ($p < 0.05$), US-CRP by 33% ($p < 0.0001$), oxygen consumption by 55% ($p < 0.0001$), and spontaneous chemiluminiscence was by 25% ($p < 0.005$) after treatment. As far as we know, this is the first study showing a chronic inflammation decrease in MetS patients due to the administration of a biopower Resveratrol-piperine and alpha tocopherol dietary supplement together with conventional therapy.

Keywords: metabolic syndrome; chronic inflammation; resveratrol; piperine; alpha-tocopherol

1. Introduction

According to the World Health Organization, obesity, a common denominator of MetS, has in general increased three-fold worldwide since 1975. Lifestyle changes, such as a marked reduction in physical activity levels and an increased consumption of high-calorie foods, have been conducive to the increased prevalence of obesity.

Epidemiological data from 2016 show that 39% of individuals over 18 were overweight and 13% were obese. It has been shown that overweight and obesity are risk factors for the noncommunicable chronic diseases (NCCDs) included in MetS. Consequently, the deterioration in quality of life for both patients and families added to the high medical and social costs are a great concern for health care systems.

MetS is a clinical disorder that is defined by the co-occurrence of other CVD risk factors, such as central obesity, abnormalities of glucose metabolism or diabetes, dyslipidemia and arterial hypertension. No standardized global data are available on the prevalence of MetS because diagnostic criteria vary in different guidelines [1]. In Argentina, a systematic review carried out with urban populations, with a mean age of 46.2, found a prevalence of MetS of 27.5% for both genders, higher in males than females, at 29.4% vs. 27.4% respectively [2].

In another systematic review conducted in Latin American countries, the prevalence reached 24.9% and was slightly higher in females (25.3%) than in males (23.2%); individuals over the age of 50 had the highest prevalence [3].

A meta-analysis conducted in China found that the general prevalence of MetS in individuals over 15 years of age was 24.5%, of which 19.2% were males and 27% were females. This prevalence increased with aging, being at around 32.4% in individuals over 60 [4].

A systematic review conducted in Brazil found an average prevalence of 29.6%, and the indigenous and rural populations were the most and the least affected with 65.3% and 14.9%, respectively [5]. In the USA, based on data from the National Health and Nutrition Examination Survey (NHANES) between 2003–2012, the prevalence of MetS was at 33%, higher in females than in males, at 35.6% vs. 30.3% respectively [6].

A systematic review conducted in Middle East countries found an average prevalence of MetS of 25% [7]. The PROMETS trial in Portugal found a prevalence of 36.5%, and MetS was slightly more common in females than in males, at 40.7% vs. 39% respectively [8].

It has been well proven that obesity features a chronic low grade inflammatory state and that the cumulative molecular damage caused by the oxidative metabolism plays a key role in the pathogenesis of age-related conditions, also contributing to increased inflammation and oxidative stress levels [9,10]. These two processes are interdependent and often share signaling pathways and biochemical processes that accelerate aging from the onset of chronic diseases included in MetS. Redox homeostasis is maintained in physiological situations at the expense of the antioxidant regulation of the concentrations of oxidant species, and in terms of molecular biology it involves intracellular protein signaling and transcription mechanisms [11]. When these mechanisms are altered or absent, an excessive accumulation of biomarkers of inflammation and oxidative stress occurs, with byproducts of biomolecular oxidation, and signaling pathways are therefore altered [11,12].

Based on these findings, several clinical trials have investigated whether vitamin antioxidants might prevent NCCDs [13,14]. However, the obtained results were generally disappointing because, although some of the studies found benefits for health, others did not obtain any results or even found deleterious effects [15]; this is because, in some cases, biology uses free radicals as signaling pathways, and massive elimination might partly account for this failure, leading to what is known as the antioxidant paradox of vitamins [16–18].

The MetS is one of the maximum expressions of chronic inflammation, as several chronic inflammatory conditions coexist. This inflammatory syndrome, which accelerates aging, is still generally underdiagnosed and undertreated in medical practice. For this reason, the aim of our research was to assess the effect of a Resveratrol + Piperine + Alpha-tocopherol (FRAMINTROL®) dietary supplement on chronic inflammation in MetS.

2. Materials and Methods

Between May and December 2019, we publicly invited out-patients with MetS to participate in a clinical trial approved by the Ethics Committee of the University of Buenos Aires, Clinics Hospital.

These included twenty-two patients (13 males and nine females), mean age 68 ± 4.7 years, diagnosed with MetS according to the global harmonized definition of having three of the five following characteristics: [19] 1. Blood glucose levels over 100 mg/dL or drug therapy for high blood glucose levels; 2. HDL Cholesterol < 40 mg/dL in males or < 50 mg/dL in women, or drug therapy for low HDL cholesterol levels; 3. Blood triglycerides > 150 mg/dL or drug therapy for high triglyceride levels; 4. Waist circumference > 102 cm for males or > 88 cm for females; and 5. Blood pressure > 130/85 mmHg or antihypertensive drug therapy.

At the first interview, the participants of this clinical trial #26122018 approved by the Ethics Committee of the "José de San Martín" Clinics Hospital University of Buenos Aires were presented, in accordance with the 1964 Helsinki Declaration and the later updated versions of Tokyo (1975), Venetia (1983), Hong Kong (1989), Somerset West (1996), Edinburgh (2000), Washington (2002), Tokyo (2004), Korea (2008) and Brazil (2013), and also according to the personal data protection Act N° 25.326; then, the inclusion criteria were analyzed, and informed consent was obtained.

The Ethics Committee, through the approved protocol, established as a condition that the background treatments for NCCDs, oral hypoglycemic agents, antihypertensives or medical therapy for dyslipidemia should not be suspended and should remain unchanged during the trial. The average baseline data of all the patients are included in Table 1.

Table 1. Patient average baseline data.

Patients (Male–Female)	22 (M 13–F 9)
Age (years)	68 ± 4.7
Weight (kg)	82 ± 17.5
Body Mass Index (kg/m^2)	29.25 ± 3.4
Systolic blood pressure (mmHg)	135 ± 25.85
Diastolic blood pressure (mmHg)	86 ± 18.32
Waist circumference (cm)	109 ± 23.30
Blood glucose (mg/dL)	103 ± 21
HDL Cholesterol (mg/dL)	57.95 ± 12.32
Triglycerides (mg/dL)	126 ± 26.86
Plasma ferritin (ng/mL)	198.45 ± 38.11
Ultrasensitive C reactive protein (mg/L)	4.10 ± 0.87
Oxygen consumption (nmol O_2/min/ 10^6 cells)	13 ± 2
Chemiluminescence (cps/mL cells)	134 ± 47

As the participation of the five different CVD risk factors within MetS varies among patients, we presented, as shown in Table 2, the percentage of each condition in MetS. Hypertension was the most prevalent entity.

Table 2. Relative participation and percentage of the five CVD risk factors in the MetS patients.

	Arterial Hypertension	**Waist Circumf**	**Triglycerides**	**HDL Cholesterol**	**High Glucose or Diabetes**	**Patients/CVD Risk Factors in MetS**
Patients	20/22 91%	19/22 86%	18/22 82%	18/22 82%	16/22 73%	10/5 5/4 7/3

Tables 3–5 show the clinical data of each patient at the beginning of the trial, where the different MetS expressions can be observed. As can be seen, some risk factors were controlled by treatment and others were not.

Table 3. Baseline data of seven MetS patients with 3/5 CVD risk factors. The shadowed cells indicate the treated conditions. BP, Blood Pressure: (mmHg); WC, waist circumference (cm); TG: Triglyderides (mg/dL); HDL Chol., HDL cholesterol (mg/dL); BG, Blood Glucose (mg/dL).

Patient ID	BP	WC	TG	HDL Col.	BG
HB	180/100	114	71	48	97
CF	120/80	88	105	77	99
MG	148/86	93	64	51	91
PH	105/78	107	90	81	106
HL	116/60	98.5	169	45	90
RS	130/90	110	90	61	90
ES	130/96	101	133	40	98

Table 4. Baseline data from five MetS patients with 4/5 CVD risk factors. The shadowed cells indicate the conditions under treatment.

Patient ID	BP	WC	TG	HDL Chol.	BG
BH	150/90	114	79	39	105
AD	160/100	99	57	77	101
LV	120/80	104	107	76	90
EV	130/80	123	282	39	89
MG	100/86	93	64	51	91

Table 5. Baseline data from 10 MetS patients with 5/5 CVD risk factors. All of them were receiving treatment for their baseline conditions.

Patient ID	BP	WC	TG	HDL Chol.	BG
JB	140/90	112	65	60	105
RB	140/92	140	131	37	136
SN	120/70	99	50	66	99
CL	160/84	128	155	79	116
JP	138/86	128	238	64	104
AS	120/80	114.5	354	30	111
MS	130/90	119	100	68	119
ET	130/90	110	202	43	110
LA	140/98	95	104	89	110
HA	170/100	103	58	54	102

Patients started treatment after a two-week washout of dietary supplements (antioxidants, vitamins or minerals), control visits were scheduled for 30, 60 and 90 days, and the treatment included two pills per day of a biopower formula of resveratrol 50 mg + piperine 5 mg + alpha tocopherol 25 mg (FRAMINTROL®), one with each main meal.

According to the design of the study, each patient was their own matched control, in order to prevent interindividual variables and the bioavailability of the active ingredient. Blood and urine tests were performed twice, at the beginning and at the end of the treatment. The following parameters in plasma were assessed: glycemia, HDL Cholesterol, triglycerides, ferritin, US CRP, and the oxygen consumption and spontaneous chemiluminescence activity in isolated neutrophils.

Biochemical measurements were obtained using a diagnostic kit from Roche Laboratories, measured with Hitach Covas C311. Hematological measurements were made using LABIX reactive kits measured with SEAC HECO.

2.1. Samples and PMN Isolation

The venous blood samples were obtained with heparin. PMNs were isolated from blood samples in the Hematology Laboratory of the Clinical Biochemistry Department of "José de San Martín" Clinics Hospital using Ficoll–Hypaque method and exposed to hypotonic shock with a sterile NaCl solution (0.2% w/v) and the same volume of sterile NaCl solution (1.6% w/v). This suspension of PMNs was centrifuged for 10 min at 450 g at 20 °C, the supernatant fluid was discarded, and the pellet was resuspended in 10 mL of sterile RPMI 1640 medium at 37 °C [20].

2.2. Neutrophils Viability

PMNs' viability was tested with a vital staining Trypan Blue solution (0.4%), and only cell suspensions with a viability >95% were used.

2.3. PMNs Count and Viability

Total and differential cell accounts of viable PMNs in suspension, were developed using the Sysmex XP-300™ Automated Hematology Analyzer (Sysmex®® XN-Series, Chuo-ku, Kobe, Japan) Sysmex Corporation, Chuo-ku, Kobe, Japan, in a mode to distinguish the mononuclear (MN) cells from neutrophils. These counts were confirmed manually with Neubauer's chamber, using a 1/400 dilution of the PMNs suspension in Turk's solution (1–2% acetic acid with aqueous methylene blue). PMNs' viability was measured with an equal volume of Trypan Blue solution (T8154-Sigma-Aldrich) using a 1/100 dilution of the abovementioned neutrophil suspension. The mononuclear cells/neutrophils ratio as well as cell viability was determined before and after each experiment. PMN samples were sent to the School of Pharmacy and Biochemistry for oxygen consumption and chemiluminescence.

2.4. PMN Oxygen Consumption

PMN oxygen uptake was determined polarographically by high resolution respirometry, using a Clark-type electrode (Hasantech Oxygraph System DW1, Norfolk, England) Hasantech Instruments Ltd, Norfolk, England, thermostated at 37 °C, with human PMN (10^6/mL) in PBS supplemented with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 7.5 mM glucose (PBSG). For the assay, respiratory buffer (for a 1 mL final volume) was placed in the electrode chamber, and the rate of oxygen consumption was calculated from the initial time course and expressed as nmol of oxygen/min/10^6 cells [21].

2.5. PMN Chemiluminescence

Spontaneous chemiluminescence in fresh PMNs was measured with a photon counter developed by Chance, Sies and Boveris (1979) in the Johnson Research Foundation of the University of Pennsylvania (Philadelphia, PA, USA). The results were expressed in cps/mL PMN (cps: counts per second, 1 cps corresponds to about 10^3 photons per second) [22–24].

2.6. Statistics

The statistical analysis was performed using the Student *t*-test for paired samples (Prim 7.0, GraphPad, San Diego, CA, USA). The Student *t*-test was selected to evaluate a single biomarker at a time in eight series of paired data (D0 vs. D90 for plasma ferritin and US CRP, in neutrophils, oxygen consumption and spontaneous chemiluminescence) for each patient before and after the intervention.

3. Results and Discussion

The 22 patients with MetS showed a good tolerance to the treatment with two pills a day of the Resveratrol 50 mg + piperine 5 mg + alpha-tocopherol 25 mg (FRAMINTROL®) formula, with no side effects.

The measurements of the inflammatory state appear in Table 6. The observed baseline and post-treatment variations were all beneficial, plasma ferritin levels decreased significantly by 10%,

and US CRP decreased in plasma by 33%, evidencing a highly significant drop (Figures 1 and 2); in neutrophils, oxygen consumption decreased by 55% and spontaneous chemiluminescence by 25% after 90 days of treatment; both decreases were highly significant (Figures 3 and 4).

Table 6. Variables of the inflammatory state; in plasma: ferritin and US CRP, and in neutrophils: baseline and post-treatment (after 90 days of resveratrol, piperine y alpha-tocopherol) oxygen consumption and chemiluminescence.

Biomarkers/ Basal vs. Final	Basal	Final	*p*	Δ %
Plasma ferritin (ng/mL)	198.45 ± 38.11	178.75 ± 21.90	<0.05	10
Oxygen consumption (nmol O_2/min/ 10^6 cells)	13 ± 2	6 ± 1	<0.0001	55
Ultrasensitive C reactive protein (mg/L)	4.10 ± 0.87	2.74 ± 0.59	<0.0001	33
Chemiluminescence (cps/mL cells)	134 ± 47	100 ± 22	<0.005	25

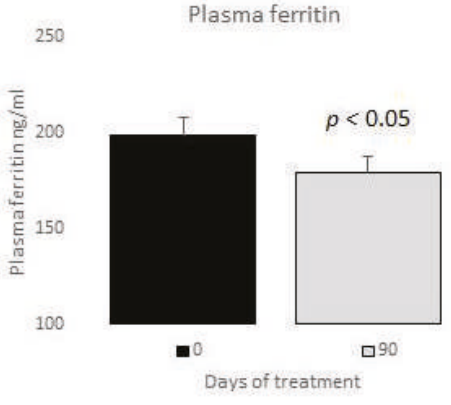

Figure 1. Plasma ferritin basal vs. 90 days of treatment with resveratrol, piperine and alpha-tocopherol in MetS patients.

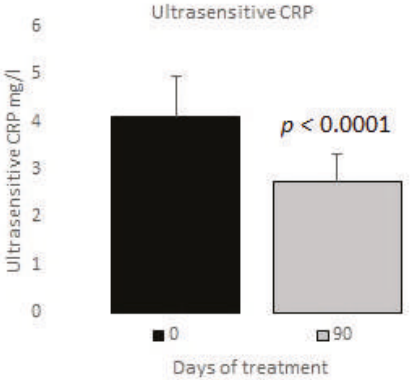

Figure 2. Ultrasensitive C reactive protein basal vs. 90 days of treatment with resveratrol, piperine and alpha-tocopherol in MetS patients.

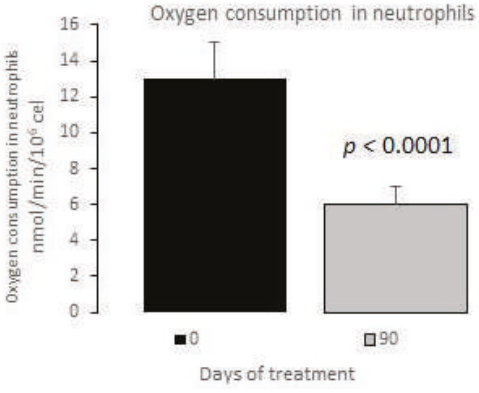

Figure 3. Oxygen consumption in neutrophils basal vs. 90 days of treatment with resveratrol, piperine and alpha-tocopherol in MetS patients.

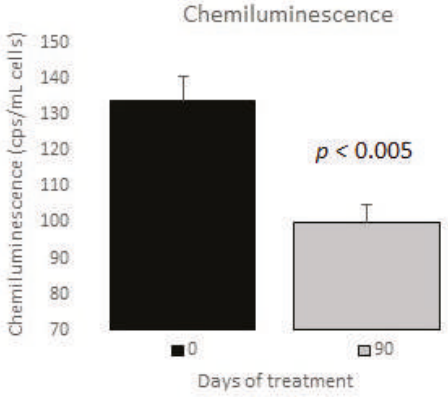

Figure 4. Chemiluminescence in neutrophils basal vs. 90 days of treatment with resveratrol, piperine and alpha-tocopherol in MetS patients.

Arterial hypertension is one of the most prevalent chronic cardiovascular conditions worldwide, and it was also the most prevalent in our MetS study population (91% of our patients) (Table 2). It has been proven that low-grade chronic inflammation in arterial hypertension is present since the onset of the disease.

Although the etiology of essential arterial hypertension is still unknown, it has been found that both the innate and adaptive immune systems participate interdependently in the development of sustained high blood pressure, as well as in endothelial, kidney and target organ damage [25].

The phenomenon that triggers arterial hypertension has not been elucidated; however, it is known that pro-hypertensive stimuli such as Angiotensin II or excessive use of salt make lymphocytes T proinflammatory, which increases the release of inflammatory cytokines as well as oxidative stress levels [26,27].

Increased metabolic activity in neutrophils in patients with MetS, as measured indirectly in oxygen consumption, might signal the intense activation of these inflammatory cells.

Obesity and diabetes were reported in 86% and 73% of the patients with MetS, respectively (Table 2). The physical effects related to the increase in adipose tissue caused by obesity provide many intrinsic signals produced by mechanical stress, hypoxia and adipocyte death that are able to initiate the inflammatory response. It has been shown that, as adipose tissue increases during

obesity, inflammation and macrophage-innate immune cell-accumulation increase as well and that this infiltration reaches as much as 40% of all the adipose tissue cells [28,29].

The mechanical stress that triggers the inflammation in adipose tissue in obese individuals was first published in the early 1990s. This investigations showed that the adipose tissue of obese volunteers presented inflammatory changes with an increased release of cytokines mediated by tumor necrosis factor-alpha (TNF-α), and this finding correlated with the inhibition of the insulin receptor substrate and consequently with an increased insulin resistance [30]. This finding has also been proven in basic research through reverse engineering, where TNF-α block increases sensitivity to insulin and improves glucose metabolism [30]. These findings confirm the significant activation of inflammatory processes by the innate and adaptive immune systems [31] in obesity, which constitute risk factors for increased insulin resistance and diabetes. In brief, lipid accumulation in adipose tissue in obese patients triggers an inflammatory response, resulting in an increased release of several cytokines [32,33]. It has also been evidenced that dyslipidemia induces an inflammatory response due to the activation of the immune system. Persistently high oxidized LDL cholesterol levels in plasma drive the production of interleukin 1 and 6 and therefore drive increases in US CRP levels [34]. High plasma ferritin is a biomarker of MetS. It was found that plasma ferritin values were correlated directly with different clinical expressions of MetS with three, four or five CVD risk factors [35,36]. In other words, there were more CVD risk factors with more plasma ferritin. Furthermore, it has been proven that, of all the MetS components, triglycerides and hyperglycemia are the variables that correlate the most with high plasma ferritin levels [37]. C reactive protein (CRP) is a protein of the pentraxin family, synthesized and released by endothelial cells in atherosclerotic plaques, hepatocytes, lymphocytes and macrophages, mainly in response to the increase of interleukin 7 (IL 7). It has been evidenced that US CRP in plasma is a biomarker of the inflammatory system in downstream MetS, and potentially a good biomarker for monitoring treatment results [12,38,39].

In our study, the post-treatment decreases in plasma ferritin and US CRP reached 10% and 33%, respectively (Figures 1 and 2). Thus, we believe that those variables are a potential tool for the clinical control of patients' outcomes.

Piperine is a bioactive alkaloid that gives black pepper its pungency. It has a broad spectrum of action in molecular biology, as an antioxidant, anti-inflammatory, antiangiogenic, antibacterial and immunomodulatory agent. Piperine may be naturally extracted from black pepper, with a 6% to 13% yield. Despite its many healthy properties, the use of piperine in human health is still limited due to its scarce availability and low solubility in water [40,41]. However, piperine has been shown to be a resveratrol booster, since the oral administration of a formula of piperine plus resveratrol (10/100 mg/kg, respectively) increases the bioavailability of resveratrol by 1544% when compared to resveratrol alone [42].

Alpha-tocopherol has been shown to have anti-inflammatory effects due to the decreased production of anti-inflammatory cytokines IL-1 beta IL-6 and chemokine IL-8, together with the neutralization of alpha-TNF [43]. In obese individuals with and without diabetes, MetS is related to chronic systemic inflammation and low levels of alpha-tocopherol [44].

Neutrophils are the main intracellular source of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) in the inflammatory processes associated with human obesity and metabolic syndrome. These reactive oxygen species are mainly generated in PMN cells through NADPH oxidase complex activity. The rate of oxygen consumption measured in the Clark-type electrode indicates the electron transfer process for generating O_2^- and, therefore, H_2O_2 production. Spontaneous chemiluminescence is a useful approach for determining the occurrence of oxidative stress in either cells or tissues. Spontaneous chemiluminescence determination is a noninvasive, nondestructive indirect assay based on measuring the light emission from an excited state to a basal state of singlet oxygen ($^1O_2$) and peroxyl radicals (ROO), reactive and toxic products of phospholipid peroxidation. The number of emitted photons depends on the steady-state concentration of $^1O_2$, which is an indicator of the oxidative damage due to the final stage of the phospholipid oxidation in the chain reaction [22–24]. Resveratrol supplementation

decreased the oxygen consumption and chemiluminescence levels in PMN of patients with MetS, indicating an anti-inflammatory and antioxidant effect after treatment (Figures 3 and 4).

4. Conclusions

Metabolic syndrome is a clinical entity with an increasing incidence and high prevalence worldwide, including several CVD risk factors; obesity, arterial hypertension, hyperglycemia and dyslipidemia have a common denominator, i.e., the chronic activation of the immune system, with inflammatory processes which damage the molecular biology and accelerate aging. In our research, we used a biopower formula containing three active ingredients: Resveratrol + Piperine + Alpha-Tocopherol (FRAMINTROL®) in the management of patients with MetS.

The results of this treatment evidenced (1) a significant decrease in plasma ferritin levels and a highly significant decrease in US-CRP levels; (2) Plasma ferritin and US CRP might be good biomarkers of inflammation for the clinical follow up of patients with MetS; (3) A highly significant decrease in the oxygen consumption and spontaneous chemiluminescence of polymorphonuclear cells might be indicative of a remarkable drop in the proinflammatory metabolism of these cells of the immune system and of decreased levels of oxidant reactive species; (4) Reducing chronic inflammation in MetS patients should be a new prevention goal to decrease CVD risk factors.

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Article

Supplementation with Red Wine Extract Increases Insulin Sensitivity and Peripheral Blood Mononuclear Sirt1 Expression in Nondiabetic Humans

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Abstract: The aim of this study was to investigate the effects of dietary supplementation with a nonalcoholic red wine extract (RWE), including resveratrol and polyphenols, on insulin sensitivity and Sirt1 expression in nondiabetic humans. The present study was a single-arm, open-label and prospective study. Twelve subjects received supplementation with RWE, including 19.2 mg resveratrol and 136 mg polyphenols, daily for 8 weeks. After 8 weeks, metabolic parameters, including glucose/lipid metabolism and inflammatory markers, were evaluated. mRNA expression of Sirt1 was evaluated in isolated peripheral blood mononuclear cells (PBMNCs). Additionally, Sirt1 and phosphorylated AMP-activated kinase (p-AMPK) expression were evaluated in cultured human monocytes (THP-1 cells). Supplementation with RWE for 8 weeks decreased the homeostasis model assessment for insulin resistance (HOMA-IR), which indicates an increase in insulin sensitivity. Serum low-density lipoprotein-cholesterol (LDL-C), triglyceride (TG) and interleukin-6 (IL-6) were significantly decreased by RWE supplementation for 8 weeks. Additionally, Sirt1 mRNA expression in isolated PBMNCs was significantly increased after 8 weeks of RWE supplementation. Moreover, the rate of increase in Sirt1 expression was positively correlated with the rate of change in HOMA-IR. The administration of RWE increased Sirt1 and p-AMPK expression in cultured THP-1 cells. Supplementation with RWE improved metabolism, such as insulin sensitivity, lipid profile and inflammation, in humans. Additionally, RWE supplementation induced an increase in Sirt1 expression in PBMNCs, which may be associated with an improvement in insulin sensitivity.

Keywords: red wine extract; resveratrol; polyphenols; insulin sensitivity; Sirt1

1. Introduction

Metabolic derangement, including type 2 diabetes mellitus (T2DM), hypertension and dyslipidemia, which is based on insulin resistance, is closely related to the initiation and progression of cardiovascular disease (CVD) [1]. Therefore, maintaining metabolic health, including improving insulin sensitivity, is important to protect vascular tissues against metabolic-derangement-related cellular damage. Individual lifestyles, including dietary habits, affect metabolic and cardiovascular health. Appropriate consumption of red wine, 20–30 g/day as amount of alcohol, is thought to be part of a healthy lifestyle [2–4]. Previous epidemiological studies have shown an inverse association between dietary polyphenol consumption and mortality from CVD [5–8]. The components of red wine contain many polyphenols, which are a complex mixture of flavonoids such as anthocyanins and flavan-3-ols

and nonflavonoids such as resveratrol, cinnamates and gallic acid [9]. Red wine polyphenols possess vasoprotective effects through anti-aggregatory platelet activity, antioxidant and anti-inflammatory properties, the generation and release of nitric oxide (NO) and glucose/lipid-metabolism-improving effects, which contribute to maintaining metabolic and cardiovascular health [10–12].

Aging is closely associated with metabolic derangement, including insulin resistance. Caloric restriction (CR)/dietary restriction (DR) retards aging or extends life spans [13]. The benefits of CR/DR for the suppression of age-related disorders, including glucose intolerance and CVD, have also been reported in rhesus monkeys and humans by improving insulin sensitivity and oxidative stress/inflammation [14–17]. Therefore, CR/DR mimetics may be anti-aging therapies, resulting in the maintenance of cardiometabolic health. Sirt1, a nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase, has been identified as one of the possible molecules through which CR/DR exerts anti-aging effects [18,19]. Resveratrol, a polyphenolic phytoalexin that occurs in red wine, has been one of the most extensively studied Sirt1 activators, as one of the CR/DR mimetics [20] and is a critical constituent that contributes to the health benefits of red wine. Thus, polyphenols, including resveratrol from red wine, may be candidates to improve cardiometabolic alterations associated with aging due to their pleiotropic properties. However, there are few reports on whether red wine polyphenols, exert beneficial effects on glucose/lipid metabolism and Sirt1 activation in humans. In this study, we investigated the effects of red wine polyphenols on glucose/lipid metabolism and Sirt1 expression in isolated peripheral blood mononuclear cells (PBMNCs) using red wine extract (RWE).

2. Materials and Methods

2.1. Composition of RWE

The alcohol-free RWE was obtained from NATURE Supplement (Osaka, Japan). This RWE is derived from red wine produced in the Rhone valley regions of southern France. The polyphenol contents in the RWE were assessed by high-performance liquid chromatography (HPLC) analysis (Figure S1) and revealed that 166 mg of wine solids contained 9.6 mg of resveratrol and 68 mg of polyphenols: catechin 1.16 mg, epicatechin 0.83 mg, tannin 29.3 mg, quercetin glycoside 0.33 mg, malvidin glycoside 1.99 mg, total anthocyanin 5.15 mg, anthocyanin monomer 4.15 mg, anthocyanin polymer 1.00 mg per 1 capsule.

2.2. Subjects and Study Design

Participants were recruited through advertisements on local posters. Males or females who were 20–70 years old were eligible. The exclusion criteria included diabetes (HbA1c $\geq$ 6.5%); pre-existing endocrine, kidney, liver, heart and malignant disease; anemia (male: hemoglobin (Hb) < 10.0 g/dL, female: Hb < 9.0 g/dL); alcohol abuse; smoking; the use of medicines/supplements; and planned lifestyle changes. We enrolled 12 participants, including 8 males and 4 females, in this study.

This study is a single-arm, open-label, prospective study and conducted at Kanazawa Medical University Hospital. Subjects were treated for 8 weeks with 2 capsules of RWE (containing 9.6 mg resveratrol and 68 mg polyphenols per capsule) twice daily for a total of 19.2 mg resveratrol and 136 mg polyphenols per day. During the study period, participants were instructed to abstain from supplements and foods suspected to contain polyphenols in significant amounts and the adherence for them was confirmed every visit. Moreover, the importance of maintaining their normal way of life was underscored. Compliance, defined as the proportion of capsules ingested relative to the intended number, was calculated when participants returned the remaining capsules during the final visit.

2.3. Overall Visits and Interventions

Examinations were performed at baseline and 4 and 8 weeks after supplementation with RWE, with the same equipment and by the same physicians. When completing the physical examination, including routine clinical biochemistry data at baseline, capsules were provided and participants were

instructed to initiate capsule consumption from the evening and twice daily. At week 4, potential adverse events were recorded and fasting blood samples were taken for safety purposes. In addition, participants visited the hospital on the examination day in the morning after overnight fasting at week 8 and then blood samples were collected.

2.4. Ethical Approval

Participants were given detailed explanations of the study protocol. Informed consent was obtained from each participant. The study protocol was approved by the Regional Committee on Health Research Ethics and the Ethical Committee of Kanazawa Medical University (IRB No. M229, Uchinada, Ishikawa, Japan) and conformed to the ethical principles set forth in the Declaration of Helsinki.

2.5. General Measurements

Body weight (BW) and body composition were measured using In Body® (Biospace Japan, Inc., Tokyo, Japan) with the participants being lightly clothed; the participants urinated during the 30 min prior to the In Body® assessment [21]. In addition, blood pressure (BP) and heart rate (HR) were measured in a sitting position after resting for 5 min [21]. Routine biochemistry and physical examinations were performed at screening to investigate the presence of exclusion criteria.

2.6. Blood Sample Analysis

Routine biochemistry (creatinine (Cr), uric acid (UA), aspartate aminotransferase (AST), alanine transaminase (ALT) and γ-glutamyl transpeptidase (γ-GTP)) parameters were analyzed continuously throughout the study at the Department of Clinical Biochemistry of Kanazawa Medical University Hospital using standard methods [21]. HbA1c and glycated albumin were measured using an automated analyzer, HLC-723® G11 (TOSHO CO., LTD., Tokyo, Japan) [21]. Serum low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein-cholesterol (HDL-C) levels were measured using enzymatic methods (QUALIGENT® HDL-C and QUALIGENT® LDL-C, SEKISUI MEDICAL. CO., LTD., Tokyo, Japan) [21]. Serum triglyceride (TG) levels were measured using enzymatic assays (Kyowa Medex, Co., Ltd., Tokyo, Japan) [21]. Free fatty acids (FFAs) were measured by a commercially available kit (Wako Chemicals, Neuss, Germany). Plasma glucose was measured in duplicate immediately after sampling on a YSI 2300 Stat Plus (YSI, Inc., Yellow Springs, OH, USA) [21]. Insulin was analyzed using a time-resolved immunofluorometric assay (AutoDELFIA Insulin kit, catalog no. B080–101, PerkinElmer, Turku, Finland) [21]. Homeostasis model assessment–insulin resistance (HOMA-IR) was calculated by the formula—fasting serum insulin (μU/mL)/fasting plasma glucose (mg/dL)/405. Serum interleukin-6 (IL-6) was measured by Human IL-6 CLEIA (Chemiluminescent Enzyme Immuno Assay) Fujirebio (Tokyo, Japan) and high-sensitivity C-reactive protein (hsCRP) was measured by a nephelometry method using N-Latex CRPII (Siemens Healthineers, Tokyo, Japan) [21].

2.7. Sirt1 mRNA Expression in Isolated Peripheral Blood Mononuclear Cells (PBMNCs)

PBMNCs were collected from 20 mL of heparinized blood at the beginning and after 8 weeks of the study and isolated using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA), as previously described [22]. PBMNCs were washed three times with phosphate-buffered saline (PBS) (−) and suspended in TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) for quantitative real-time PCR. Total RNA was isolated from isolated PBMNCs, cDNA synthesis and quantitative real-time PCR were performed as previously described [22]. TaqMan probes for Sirt1 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The analytical data were adjusted to the level of 18S mRNA expression as an internal control.

2.8. THP-1 Cell Culture

Human monocytes (THP-1 cells) obtained from ATCC were cultured in RPMI medium with 10% fetal calf serum [23]. After 16 h of serum starvation, THP-1 cells were treated with RWE 166, 332 and 3320 ng/mL (including 68, 136 or 1360 ng/mL polyphenols and 9.6, 19.2 and 192 ng/mL resveratrol, respectively) or Dimethyl sulfoxide (DEMSO) as a control for 24 h. Western blotting was performed using antibodies against Sirt1 (1:1000), phosphor(p)-AMPKα (Thr 172) (1:1000), AMPKα (1:1000) and β-actin (1:1000), as previously described [23]. The anti-rabbit polyclonal p62 antibody (PM045) was obtained from Medical & Biological Laboratories (Nagoya, Japan). Anti-phospho (p)-AMPKα (Thr 172), AMPKα and β-actin antibodies were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA) and anti-Sirt1 antibodies were obtained from Millipore (Bedford, MA, USA).

2.9. Statistical Analysis

Data are presented as the means ± the standard deviation (SD) unless otherwise indicated. The results obtained at baseline and after 8 weeks of RWE supplementation, as well as changes within a group, were compared using a paired *t*-test. One-way ANOVA followed by Tukey's multiple comparison test was used to determine the significance of pairwise differences among three or more groups. The correlation of two variables was analyzed by a single linear regression analysis as a Pearson correlation coefficient. Statistical significance was defined as $p < 0.05$ and statistical analyses were performed using StatMate5.

3. Results

3.1. Characteristics at Baseline and after Supplementation with RWE for 8 Weeks

The physical characteristics of the participants are shown in Table 1. BW and body mass index (BMI) were not significantly different between the baseline and the end of supplementation with RWE. Body composition, including fat mass, %fat and skeletal fat, showed no significant change between baseline and at 8 weeks of RWE supplementation. Systolic and diastolic BP and HR also showed no change between baseline and after supplementation with RWE. Fasting plasma glucose and serum insulin levels showed no significant change between baseline and the end of RWE supplementation (Table 2). However, HOMA-IR was significantly decreased after supplementation with RWE compared to the baseline. Additionally, the levels of serum TG and LDL-C were significantly decreased and serum HDL-C and FFA levels showed no differences after supplementation with RWE compared to those at baseline. Among the inflammatory markers, serum hsCRP levels were not changed; however, serum IL-6 levels showed significant decreases after 8 weeks of supplementation with RWE from baseline (Table 2). Liver function tests, such as AST, ALT and γ-GTP and kidney function tests, such as serum creatinine and uric acid, exhibited no significant change between baseline and the end of supplementation with RWE.

Table 1. Characteristics of participants at baseline and after supplementation with red wine extract.

	0 Week	8 Weeks	*p* Value
Age	47.5 ± 11.3		
Male:female	8:4		
Body weight (kg)	66.6 ± 16.4	66.0 ± 16.1	0.282
Body mass index (kg/m^2)	23.3 ± 3.8	23.1 ± 3.7	0.278
Systolic blood pressure (mmHg)	118.8 ± 13.5	119.7 ± 13.3	0.658
Diastolic blood pressure (mmHg)	71.5 ± 10.4	73.3 ± 13.0	0.580
Heart rate (/min)	71 ± 8.7	71.4 ± 5.2	0.860
Fat mass (kg)	18.8 ± 8.8	17.8 ± 8.4	0.067
%fat	25.7 ± 9.3	24.1 ± 7.0	0.255
Skeletal muscle mass (kg)	26.1 ± 6.4	26.5 ± 6.8	0.369

Table 2. Laboratory data of participants at baseline and after supplementation with red wine extract.

	0 Week	8 Weeks	*p* Value
Fasting plasma glucose (mg/dL)	95.6 ± 8.2	89.6 ± 9.3	0.100
Fasting serum insulin (μU/mL)	7.06 ± 5.49	4.88 ± 3.88	0.063
HOMA-IR	1.71 ± 1.38	1.13 ± 1.03	0.046
HbA1c (%)	5.2 ± 0.5	5.2 ± 0.4	0.135
Glycated albumin (%)	13.8 ± 1.2	13.8 ± 1.1	0.431
LDL-C (mg/dL)	119.7 ± 21.0	114.7 ± 19.6	0.013
HDL-C (mg/dL)	56.0 ± 19.0	58.0 ± 11.3	0.097
TG (mg/dL)	246.9 ± 285.6	182.2 ± 220.5	0.032
log-TG	2.12 ± 0.48	2.02 ± 0.40	0.034
Free fatty acid	515.0 ± 309.8	549.5 ± 177.6	0.688
log free fatty acid	2.62 ± 0.30	2.72 ± 0.16	0.305
IL-6 (ng/mL)	1.8 ± 0.8	1.4 ± 0.6	0.019
hsCRP (mg/dL)	1049.7 ± 1620.2	1158.3 ± 1638.6	0.400
log-hsCRP	2.70 ± 0.52	2.73 ± 0.58	0.666
AST (IU/mL)	21.3 ± 7.3	21.3 ± 7.4	0.352
ALT (IU/mL)	17.0 ± 18.7	20.0 ± 20.8	1.000
γ-GTP (IU/mL)	22.3 ± 71.1	24.0 ± 59.2	0.435
Cr (mg/dL)	0.72 ± 0.16	0.69 ± 0.15	0.054
Uric acid (mg/dL)	6.1 ± 2.2	6.1 ± 2.0	0.574

HOMA-IR: homeostasis model assessment–insulin resistance, LDL-C: low-density lipoprotein-cholesterol, HDL-C: high-density lipoprotein-cholesterol, TG: triglyceride, IL-6: interleukin-6, hsCRP: high-sensitivity C-reactive protein, AST: aspartate aminotransferase, ALT: alanine transaminase, γ-GTP: γ-glutamyl transpeptidase, Cr: creatinine, UA: uric acid.

3.2. Change in Sirt1 Expression in Isolated PBMNCs after Supplementation with RWE and the Relationship with the Change in HOMA-IR

Supplementation with RWE for 8 weeks significantly increased Sirt1 mRNA expression in isolated PBMNCs compared to baseline (Figure 1A). Additionally, the relationship between the rate of change in Sirt1 expression in isolated PBMNCs (Δ%Sirt1 mRNA expression) and the rate of change in HOMA-IR (Δ%HOMA-IR) from Pearson's correlation coefficient analysis showed a positive correlation (r = 0.6518, $p = 0.0216$) between baseline and the end of RWE supplementation (Figure 1B).

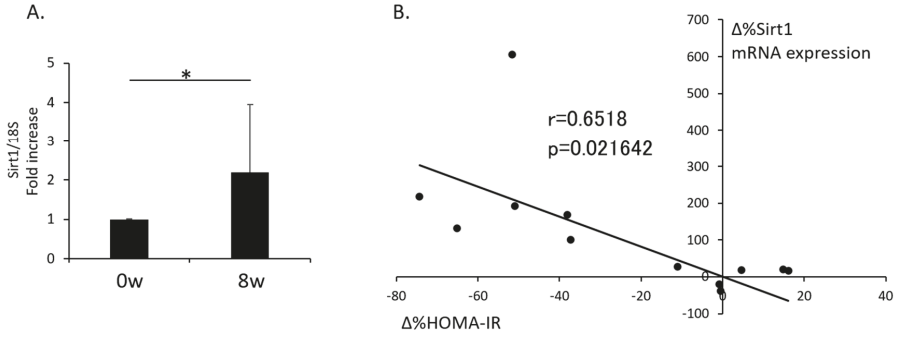

Figure 1. Change in mRNA expression of Sirt1 in PBMNCs after supplementation with red wine extract and the relationship between the change in Sirt1 expression and insulin sensitivity. (**A**) mRNA expression of Sirt1 normalized to 18S levels in isolated PBMNCs ($n = 12$). The data shown are the means ± the standard deviations. * $p < 0.05$ vs. the indicated groups. (**B**) The relationship between the rate of change in Sirt1 expression in isolated PBMNCs (Δ%Sirt1 mRNA expression) and the rate of change in HOMA-IR (Δ%HOMA-IR) from Pearson's correlation coefficient analysis ($n = 12$). PBMNCs: peripheral blood mononuclear cells, HOMA-IR: homeostasis model assessment–insulin resistance.

3.3. RWE Increased Sirt1 and p-AMPK Expression in Cultured THP-1 Cells

We evaluated whether RWE induced Sirt1 and p-AMPK expression in cultured human THP-1 cells. The administration of RWE at 166, 332 and 3320 ng/mL (including 68, 136 or 1360 ng/mL polyphenols and 9.6, 19.2 and 192 ng/mL resveratrol) in cultured THP-1 cells for 24 h significantly increased both Sirt1 and p-AMPK expression (Figure 2A–C). In addition, we confirmed that those RWE concentrations were non-toxic to cultured THP-1 cells by (data not shown).

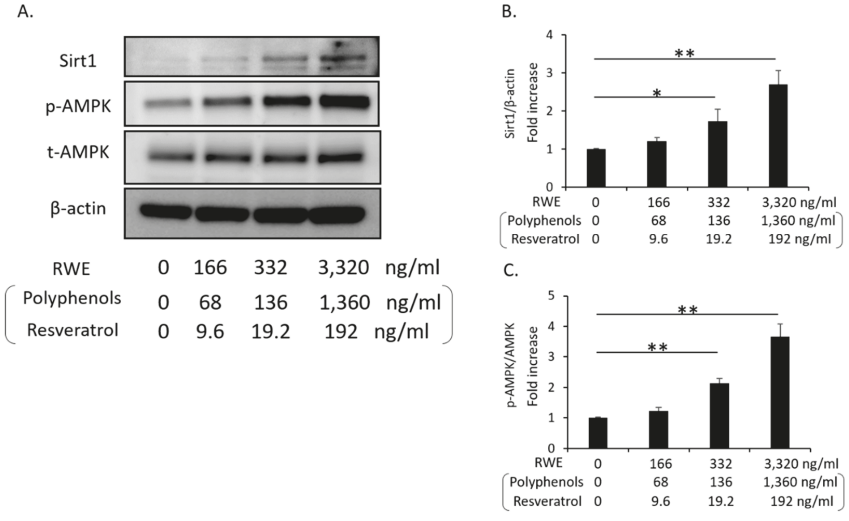

Figure 2. Change in Sirt1 and p-AMPK expression by the administration of red wine extract in cultured THP-1 cells. (**A**) Representative western blots of Sirt1, p-AMPK, AMPK and β-actin in cultured THP-1 cells ($n = 4$). (**B**) Quantitative ratios of Sirt1 to β-actin ($n = 4$). (**C**) Quantitative ratios of p-AMPK to AMPK ($n = 4$). The data shown are the means ± the standard deviations. * $p < 0.05$, ** $p < 0.01$ vs. the indicated groups. AMPK: AMP-activated kinase, RWE: red wine extract.

4. Discussion

In this study, we demonstrated that supplementation with RWE for 8 weeks significantly increased insulin sensitivity, which was evaluated by HOMA-IR in humans. Additionally, after supplementation with RWE, serum IL-6 concentration was significantly reduced and showed a decrease in the levels of LDL-C and TG. Moreover, RWE supplementation enhanced Sirt1 expression in isolated PBMNCs, which was associated with an increase in insulin sensitivity.

Previous clinical evidence suggests that red wine consumption exerts beneficial effects on glucose metabolism, including insulin sensitivity. Da Luz et al. showed that regular red wine drinkers (at least one glass of red wine 4–5 days/week for 5 years) have lower glucose levels and a lower occurrence of diabetes than abstainers [24]. Additionally, Napoli et al. demonstrated that red wine consumption (360 mL/day) for 2 weeks markedly improved insulin resistance in patients with T2DM compared to the control group [25]. Chiva-Blanch et al. also compared the effect of moderate consumption of red wine (30 g alcohol/day), dealcoholized red wine and gin on glucose metabolism in 67 men with high cardiovascular disease for 4 weeks [26]. Red wine and dealcoholized red wine but not gin exhibited decreases in plasma insulin levels and HOMA-IR [26]. In this study, we also demonstrated that the values of HOMA-IR were significantly reduced after the administration of RWE containing 136 mg polyphenols per day for 8 weeks in nondiabetic humans.

Since red wine is rich in polyphenolic compounds, including flavonoids (anthocyanins, tannins and catechin) and nonflavonoids (stilbenes such as resveratrol, tyrosol and hydroxytyrosol) [9], the beneficial

effects of red wine are thought to be exerted through polyphenols. Among the polyphenols, resveratrol has been one of the most extensively studied as a critical constituent that contributes to the health benefits of red wine. Previous studies demonstrated that resveratrol might play potential therapeutic roles in cardiometabolic health through multiple mechanisms, such as anti-inflammatory, antioxidant and anti-diabetic effects, which are mediated by the activation of Sirt1, estrogen receptor (ER) signaling, nuclear factor-erythroid-derived 2-related factor-2 (Nrf2) or AMPK [20,27–29]. Several reports showed that catechin, epicatechin, quercetin and anthocyanin also can activate Sirt1 or AMPK [30–33], however, the number of reports is so few, compared to those of resveratrol. In this study, we focused on Sirt1, which is an important regulator of a wide variety of cellular processes, including glucose/lipid metabolism and anti-inflammation, via the deacetylation of many substrates [34,35]. Our data showed the increased expression of Sirt1 in PBMNCs after supplementation with RWE including 19.2 mg resveratrol. Additionally, the levels of Sirt1 expression in PBMNCs had a positive relationship with insulin sensitivity, which was evaluated by HOMA-IR. Additionally, serum IL-6 was reduced after the administration of RWE including resveratrol. A previous report showed that decreased Sirt1 expression levels in circulating monocytes are correlated with insulin resistance in humans [36]. Moreover, Gillum et al. reported that Sirt1 expression was reduced in adipose tissues of obese males with insulin resistance and mRNA expression of CD14, a macrophage marker, in adipose tissue is negatively correlated with Sirt1 expression [37]. Chronic low-grade tissue inflammation is an important etiological component of insulin resistance [38]. Elevated levels of proinflammatory cytokines, such as IL-6, in the blood have been detected in individuals with insulin resistance. The activation of monocytes/macrophages in the circulation and adipose tissue has been demonstrated to lead to the release of various inflammatory mediators. Sirt1 may contribute to the negative regulation of inflammation in several tissues or cells, including monocytes/macrophages, through the deacetylation of NF-κB (p65 subunit) [39–41]. Therefore, the effect of RWE including resveratrol on insulin resistance and inflammation may be associated with increased Sirt1 expression in PBMNCs. However, we could not show the levels of acetylated NF-κB (p65) in mononuclear cells or the relationship between Sirt1 expression and serum IL-6 values.

In addition to reducing inflammation, previous reports showed that Sirt1 may positively regulate insulin signaling by interacting with tyrosine phosphatase 1B, insulin receptor substrate or phosphoinositide 3-kinase in insulin-sensitive tissues such as skeletal muscle [42–44]. Timmers et al. also reported that resveratrol supplementation (150 mg/day) for 30 days in obese humans increased insulin sensitivity, improved muscle mitochondrial respiration and activated Sirt1 and AMPK in skeletal muscle [45]. Additionally, Liu et al. reported that resveratrol inhibited inflammation and ameliorated insulin-resistant endothelial dysfunction through AMPK and Sirt1 [46]. In this study, we demonstrated that the administration of RWE including resveratrol increased the expression of Sirt1 and p-AMPK in cultured THP-1 cells. However, we could not evaluate Sirt1 expression levels or inflammation in other tissues/cells, such as skeletal muscle, adipose tissue or endothelial cells.

On the other hand, other reports indicate that resveratrol has no effects on insulin sensitivity. Yoshino et al. demonstrated that oral resveratrol (75 mg/day) supplementation in nonobese and postmenopausal women with normal glucose tolerance did not improve metabolic function, including insulin sensitivity [47]. Moreover, Poulsen et al. reported that resveratrol (500 mg/day) supplementation in obese men had no effect on insulin sensitivity [48]. Thus, the efficacy of resveratrol for insulin sensitivity is controversial in humans. Therefore, the beneficial effects of RWE may be attributed to the overall mix of all of its components and not to a specific action of one, such as resveratrol.

Sirt1 regulates lipid metabolism through the modulation of sterol regulatory element-binding protein (SREBP)-1C activity, liver X-receptor (LXR) and farnesoid X receptor (FXR) via deacetylation of those molecules [49–51]. Therefore, in this study, RWE including resveratrol might contribute to deceased levels of LDL-C and TG through Sirt1 activation, in addition to increased insulin sensitivity.

There are several limitations in this study. First, this study is a single-arm, open-label study with small sample size and occurred over a short time period. Second, participants in this study are

individuals who are interested in health and the supplements. Therefore, the bias on the results in this study cannot be eliminated. However, the results from in vitro experiments showing that RWE increases Sirt1 expression can support the results in PBMNCs of this study. Third, we could not measure the concentration of polyphenols, including resveratrol, in circulation. Fourth, we evaluated Sirt1 expression in only PBMNCs, not in other tissues/cells. Lastly, we evaluated insulin resistance only by the calculation of HOMA-IR, although the gold standard method for assessment of insulin sensitivity is a hyperinsulinemic-euglycemic clamp study.

5. Conclusions

Supplementation with RWE improved metabolism, such as insulin sensitivity, lipid profile and inflammation, in nondiabetic humans. Additionally, RWE supplementation induced an increase in Sirt1 expression in PBMNCs, which may be associated with an improvement in insulin sensitivity. However, further study including a randomized control trial or a cross over trial will be required to confirm these results.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/10/3108/s1, Figure S1: Analysis of red wine extract by HPLC.

Author Contributions: M.K. and D.K. designed the study, researched and analyzed the data and wrote and edited the manuscript. M.K., Y.O. and D.K. contributed to the research and to the collection and analysis of the data. I.M. contributed to the discussion. M.K. and D.K. are the guarantors of this work. All authors have read and agreed to the published version of the manuscript.

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Postprandial Effect of Yogurt Enriched with Anthocyanins from Riceberry Rice on Glycemic Response and Antioxidant Capacity in Healthy Adults

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Abstract: The pigment of riceberry rice has been reported to contain anthocyanins which act as a free radical scavenger and inhibitor of carbohydrate digestive enzymes. Since the probiotic yogurt incorporated with the pigment of riceberry rice extract was previously developed, the present study was aimed to investigate the acute effect of riceberry rice yogurt consumption on postprandial glycemic response, antioxidant capacity, and subjective ratings in healthy adults. In a cross-over design, 19 healthy participants were randomized to consume 350 g of yogurt supplemented with 0.25% *(w/w)* riceberry rice extract or the control yogurt. Postprandial plasma glucose, antioxidant status, and subjective ratings were measured at fasting and intervals (0–3 h) after ingestion of yogurt. The primary outcome was glycemic response; the secondary outcomes were plasma antioxidant capacity. In comparison to the yogurt control, riceberry rice yogurt reduced plasma glucose concentration after 30 min of consumption. The incremental area under the curve (iAUC) was significantly lower after riceberry rice yogurt load than after the control yogurt load. The consumption of riceberry yogurt caused an acute increase in plasma ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), and oxygen radical absorbance capacity (ORAC) from the baseline values after 60 min of 0.25 ± 0.06 mM $FeSO_4$, 253.7 ± 35.5 mM Trolox equivalents, and 166.8 ± 28.9 mM Trolox equivalents, respectively. Furthermore, the iAUCs for FRAP, TEAC, ORAC, and protein thiol were higher in riceberry yogurt consumption compared with the control yogurt (1.6-, 1.6-, 2.9-, and 1.9-fold, respectively). A decrease in iAUC for plasma malondialdehyde (MDA) concentration was also observed in the riceberry yogurt group. However, consumption of riceberry rice yogurt and control yogurt showed similar subjective rating scores of hunger, desire to eat, fullness, and satiety. In conclusion, acute consumption of riceberry rice yogurt suppressed postprandial glucose level and improved plasma antioxidant capacity in healthy volunteers.

Keywords: yogurt; riceberry rice; postprandial; glycemia; antioxidant; anthocyanin

1. Introduction

Yogurt, a semisolid fermented milk produced by lactic acid bacteria, is considered as an important functional food because of high nutritional content such as protein, calcium, vitamin B, phosphorus, magnesium, and potassium [1]. Recent studies reveal that consumption of yogurt fermented by lactic acid bacteria improves gastrointestinal health mediated through gut microflora, bowel transit, and immune response [2]. Especially, probiotic bacteria in yogurt have beneficial effects on host's health by conferring protection against pathogenic bacteria and the prevention of gastrointestinal disorder such

as irritable bowel syndrome (IBS), acute infectious diarrhea, or food intolerance [3,4]. In long-term intervention studies, the consumption of yogurt has been demonstrated to reduce the risk of developing metabolic diseases such as type 2 diabetes and cardiovascular diseases [5,6]. Interestingly, an intake of probiotic yogurt markedly improved glycemic control and increased antioxidant status in patient with type 2 diabetes [7].

In recent years, the role of certain plant-based foods on health benefit has drawn attention to consumers. Following this trend, several attempts have been made to the manufacture of yogurt containing the natural extract from fruits and vegetables in order to improve its nutritional, biological, and sensory properties [8]. For example, addition of cherries, berries, and grapes to yogurts increased the content of bioactive compounds such as phenolic acids and polyphenols [8,9]. Moreover, yogurt fortified with anthocyanins from chokeberries increased antioxidant activity and improved acceptable sensory properties [10]. Interestingly, combination of yogurt and plant-based foods also enhanced proliferation and survival of probiotics and modulated human microbiome [11,12].

Riceberry rice (*Oryza sativa* L.), a grain covered with deep-purple pigment, contains many nutrient components and bioactive compounds including anthocyanins, mainly cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) [13]. It has been shown that the extract from riceberry rice possesses various biological activities such as antioxidant, anti-hyperglycemic, anti-hyperlipidemic, anti-glycation, and anti-inflammatory activities [13,14]. Consumption of riceberry rice bread had lower glycemic response with increased antioxidant capacity in healthy subjects when compared to Hom mali rice bread [15]. The probiotic yogurt enriched with anthocyanin-rich extract from riceberry rice was successfully developed and improved its functionality by increasing the amount of total phenolic compounds and antioxidant activity [16]. However, there is a substantial lack of evidence on postprandial glycemic response and antioxidant capacity of riceberry rice yogurt consumption in humans. Therefore, this study was aimed to investigated whether the consumption of riceberry rice yogurt affect the level of postprandial plasma glucose in healthy volunteers. The study was also investigated the effect of riceberry rice yogurt on plasma antioxidant capacity, the marker of lipid peroxidation and subjective appetite sensations.

2. Materials and Methods

2.1. Ethical Approval

The study was approved by the office of Ethics Review Committee for Research Involving Human Research Subjects, Human Science Group, Chulalongkorn University (COA No. 241/2018). The study began in January 2019 and completed in March 2019. The trial was registered at the Thai Clinical Trials Registry (study ID: TCTR20190118006). All subjects gave their written informed consent to participate. All information of participants was kept confidential. There were no major changes in the study protocol after initiation of the study.

2.2. Riceberry Rice Yogurt

The set-type yogurt was obtained from the report of Anuyahong et al. [16]. The ingredients consisted of whole milk, skimmed milk powder (3% *w/w*), and sucrose (5% *w/w*), with or without the anthocyanin-rich extract of riceberry rice (0.25% *w/w*). The yogurt contained *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* subsp. *lactis* BB-12. The proximate analysis of yogurt was performed by The Food Research and Testing Laboratory, Faculty of Science, Chulalongkorn University. The nutritional and phytochemical composition are reported in Table 1.

Table 1. The nutritional composition and phytochemical compounds of yogurt for one serving size (350 g).

Composition	Control Yogurt	Riceberry Rice Yogurt
Energy (kcal)	349.1	349.6
Carbohydrate (g)	43.5	43.9
Total dietary fiber	n.d	n.d
Protein (g)	13.8	13.8
Fat (g)	13.3	13.2
Moisture (g)	275.9	275.5
Ash (g)	3.5	3.6
Total polyphenol content Anthocyanins	16.1	28.1
Cyanidin-3-glucoside	n.d	17.4
Peonidin-3-glucoside	n.d	7.9

n.d = not detected.

2.3. Participants

Twenty-three subjects were recruited to participate in this study. The inclusion questionnaire was used to evaluate the eligibility criteria of participants. The inclusion criteria were as follow: (1) age of 18–40 years; (2) body mass index (BMI) of 18.5–22.9 kg/m^2; (3) fasting blood glucose level < 100 mg/dL; (4) total cholesterol level < 200 mg/dL; (5) triglyceride level < 150 mg/dL; and (6) physically active. They were excluded if they met any of the following criteria: (1) pregnant or lactating; (2) presence of diabetes mellitus or insulin resistance; (3) use of dietary supplement or medication know to interfere with glucose homeostasis; (4) self-report of alcohol or tobacco products; and (5) allergy or intolerance to yogurt or dairy products. From this group, 23 subjects met the inclusion criteria.

2.4. Study Design

The study was a randomized-crossover trial with a one-week washout period. The subjects were randomly assigned to the ingestion of yogurt supplemented with or without 0.25% riceberry rice extract according to a sequence of random numbers, obtained from the online random number generator (www.randomizer.org). The process of randomization was performed by a principle researcher with a concealed allocation design. The participants were instructed not to take foods high in phytochemicals at least three days before each study period. Additionally, they were also asked to maintain stable habitual dietary intake and activity throughout their participation in the study period and to refrain from alcohol intake and heavy exercise for 24 h before each test. The primary outcome was glycemic response (postprandial glucose). The secondary outcomes were plasma antioxidant capacity (the ferric reducing ability of plasma, the Trolox equivalent antioxidant capacity, the oxygen radical absorbance capacity, the thiol group, and plasma malondialdehyde).

On the day of testing, the participants arrived at the Department of Nutrition and Dietetics, Faculty of Allied Health Sciences, Chulalongkorn University after overnight fast. Anthropometrics (body weight and height) were measured upon arrival at each session. Following baseline measurements, the fasting blood sample was collected from forearm vein with intravenous catheter by a registered nurse. Thereafter, the subjects were asked to the rate of hunger, fullness, desire to eat, and satiety with a visual analog scale (VAS) rating using a 10-cm scale from 0 ("not at all") to 10 ("extremely") at 0 min (before). They completely consumed 350 g of the study yogurt within a 10-min period. Blood samples were drawn at 15, 30, 60, 90, 120, 150, and 180 min, while subjective ratings were taken at 30, 60, 90, 120, 150, and 180 min postprandially. The subjects were restricted to drink water (<500 mL) during the study.

2.5. Biochemical Analysis

The blood samples were collected into blood colleting tube containing sodium fluoride and EDTA as anticoagulants for the measurement of glucose and antioxidant capacity, respectively. The plasma samples were separated by centrifuged at 3000 rpm for 15 min at 4 °C and kept at −80 °C for further analysis. The level of plasma glucose concentration was measured by glucose oxidase assay according to the manufacturer's protocol (Glucose Liquicolor, HUMAN GmbH, Wiesbaden, Germany).

The ferric reducing ability of plasma (FRAP) assay in a redox-linked colorimetric reaction was performed according to a previous published report [17]. In brief, the plasma sample (10 µL) was mixed with 90 µL of freshly prepared FRAP reagent (0.3 M sodium acetate buffer (pH 3.6), 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, and 20 mM $FeCl_3$). After incubation for 30 min at room temperature, the absorbance was read at 595 nm. The results were expressed as the EC (Equivalence concentration) value obtained from a standard curve of $FeSO_4$.

The Trolox equivalent antioxidant capacity (TEAC) assay was measured using 2,2'-azinobis (3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) radical ($ABTS^{\bullet+}$) [15], which was prepared by mixing 7 mM ABTS in 0.1 M PBS (pH 7.4) together with 2.45 mM $K_2S_2O_8$ in distilled water (1:1, *v/v)*. After incubation for 16 h at room temperature, the $ABTS^{\bullet+}$ solution was diluted with 0.1 M PBS (pH 7.4) to adjust the absorbance between 0.9 and 1.0 at 734 nm. The plasma (10 µL) was incubated with the adjusted $ABTS^{\bullet+}$ solution (90 µL) for 6 min at the room temperature. The absorbance was recorded at 734 nm. The result of plasma TEAC was expressed as mM Trolox equivalents.

The oxygen radical absorbance capacity (ORAC) assay was performed according to a previous report [18]. Briefly, the 10× dilution of plasma in 0.1 M phosphate buffer saline (PBS), pH 7.4 (25 µL) was incubated with 4.8 nM sodium fluorescein in 75 mM PBS (150 µL) at 37 °C. After 10 min of incubation, 64 mM AAPH (25 µL) was added to the mixture. The fluorescence intensity was measured for 1 h with 2-min interval at excitation 485 nm and emission 535 nm. The ORAC value was calculated from the area under the curve (AUC) and expressed as µmol Trolox equivalents.

The thiol group level in plasma was measured using an Ellman's assay [19] with slight modification. In brief, plasma sample (90 µL) was mixed with 130 µL of 2.5 mM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in 0.1 M PBS, pH 7.4 and incubated for 15 min at the room temperature. Then, the absorbance was measured at 410 nm. The plasma thiol level was calculated and expressed as µM L-cysteine equivalent.

Plasma malondialdehyde (MDA), a lipid peroxidation product, was quantified using thiobarbituric acid reactive substances (TBARS) assay. The plasma sample (200 µL) was mixed with trichloroacetic acid (10% *w/v*) and 50 mM 2,6-Di-tert-bytyl-4-methylphenol (BHT) and centrifuged at 12,000 rpm for 10 min [15]. The supernatant (200 µL) was separated and further mixed with 0.67% TBA before boiling at 100 °C for 10 min. After cooling down to room temperature, the absorbance of pink-colored of reaction was measured at 532 nm. Plasma MDA concentration was calculated from the calibration curve of MDA and expressed as µmol/L MDA.

2.6. Sample Size

The sample size was calculated following a previous study of postprandial response that reported a significant change in AUC for plasma glucose with a study power of 80% and alpha of 0.05 [20]. The sample size of 17 subjects were calculated with a confidence level of 95% (α = 5%) and power of 80%. Considering the 30% dropout, the final sample size was increased to 23 subjects.

2.7. Statistical Analysis

The results are expressed as mean ± SEM. The normality and homogeneity of the data was tested by Shapiro–Wilk test. Repeated-measures ANOVA was performed to determine the effect of treatment, time, and interaction of treatment and time followed by a paired *t* test to find the significance for each time point at $p < 0.05$. The incremental area under the curve (iAUC) for postprandial glucose,

antioxidant status and lipid peroxidation (MDA) was calculated by using the trapezoidal rule integrated count areas above and below the fasting baseline concentration. Paired samples t-tests confirmed significant differences in the results from iAUC ($p < 0.05$).

3. Results

3.1. Participants

Twenty-three participants were recruited at the beginning and only nineteen participants (8 male and 11 female) completed the study. Four participants who did not receive intervention were excluded from analysis. The recruitment and enrollment data are presented in Figure 1. The characteristics of participants are reported in Table 2.

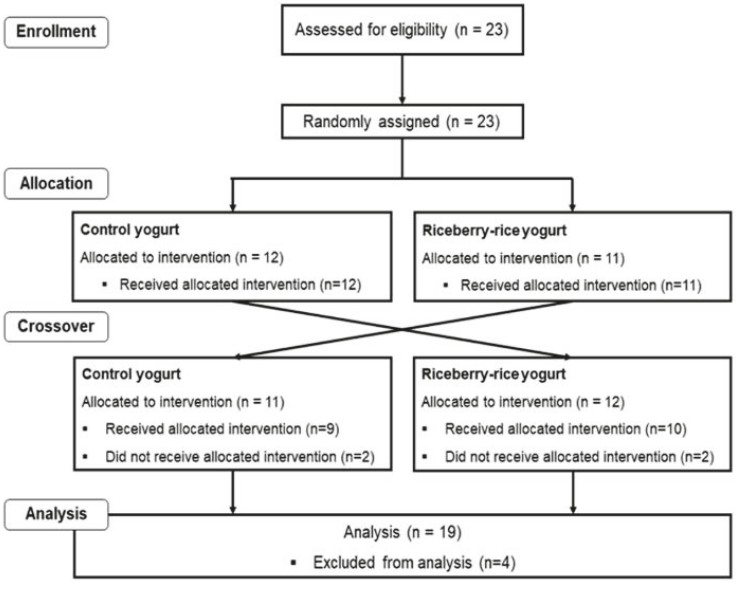

Figure 1. Consolidating Standards of Reporting (CONSORT) flow diagram of selection of study participants.

Table 2. Baseline characteristics of participants.

Parameters	Mean ± SEM
Age (years)	28.1 ± 3.0
Height (m)	1.65 ± 0.1
Weight (kg	58.3 ± 2.2
BMI (kg/m^2)	21.2 ± 0.4
Fasting plasma glucose (mg/dL)	83 ± 1.0
Total cholesterol (mg/dL)	167.3 ± 8.1
Serum triglyceride (mg/dL)	53.0 ± 3.8
LDL-C (mg/dL)	113.5 ± 6.6
HDL-C (mg/dL)	43.2 ± 3.5
Creatinine (mg/dL)	0.7 ± 0.1
Blood urea nitrogen (BUN) (mg/dL)	11.6 ± 0.5

All values are mean ± SEM, $n = 19$.

3.2. Postprandial Plasma Glucose

Incremental changes in postprandial plasma glucose concentration after consumption of yogurts are demonstrated in Figure 2A. The peak of plasma glucose level was at 15 and 30 min after consumption of all yogurts. Postprandial plasma glucose concentration following riceberry yogurt was significantly lower than following the control at 30 and 120 min. As shown in Figure 2B, a reduction in iAUC of glucose for riceberry rice yogurt relative to the control was observed.

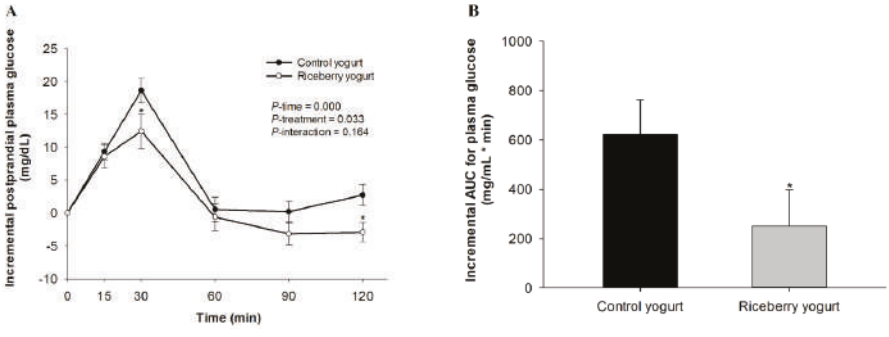

Figure 2. Incremental changes in postprandial plasma glucose concentration (**A**) and the incremental area under the curve (iAUC) for postprandial plasma glucose (**B**) in healthy participants after consuming either the control yogurt (●) or riceberry rice yogurt (○). Data are presented as mean ± SEM, $n = 19$. * $p < 0.05$ compared to the control yogurt.

3.3. Postprandial Plasma Antioxidant Status

Compared with the control, the incremental changes in postprandial FRAP was significantly increased at 60, 90, and 120 min after consumption of riceberry rice yogurt (Figure 3A). The results show that consumption of riceberry rice yogurt resulted 1.6-fold greater in the iAUC of plasma FRAP level when compared with the control (Figure 3B).

Consumption of all yogurts caused a significant increase in TEAC above baseline for all time points. Plasma TEAC level at 30, 90, 120, and 150 min was significantly higher in participants who received riceberry rice yogurt than in those who consumed the control yogurt (Figure 3C). The iAUC of postprandial plasma TEAC was 1.6-fold greater in riceberry rice yogurt than in the control (Figure 3D).

Incremental changes in postprandial plasma ORAC after consumption of yogurts are presented in Figure 3E. The postprandial plasma ORAC level appeared immediately following intake of all yogurts and returned to the baseline level at 180 min. The plasma ORAC level was significantly higher for riceberry rice yogurt than the control at 30, 60, 90, and 120 min. In particular, the 2.9-fold increase in iAUC of plasma ORAC was observed for the subjects who consumed riceberry rice yogurt (Figure 3F).

Figure 3G presents incremental changes in postprandial plasma thiol after ingestion of yogurts. The postprandial plasma thiol level was elevated after consumption of all yogurts when compared to the baseline level. The results show that the postprandial plasma thiol level did not differ between riceberry rice yogurt and the control at each time point. Nevertheless, riceberry rice yogurt caused a 1.8-fold increase iAUC of plasma thiol, in comparison to the control yogurt (Figure 3H).

Incremental changes in postprandial plasma MDA concentration after consumption of yogurts are shown in Figure 3I. Plasma MDA concentration increased significantly from baseline following the control yogurt. Interestingly, consumption of riceberry rice yogurt markedly reduced the rise in plasma MDA concentration at 60, 90, and 180 min. A reduction in iAUC of postprandial plasma MDA (33%) was perceived following consumption of riceberry rice yogurt (Figure 3J).

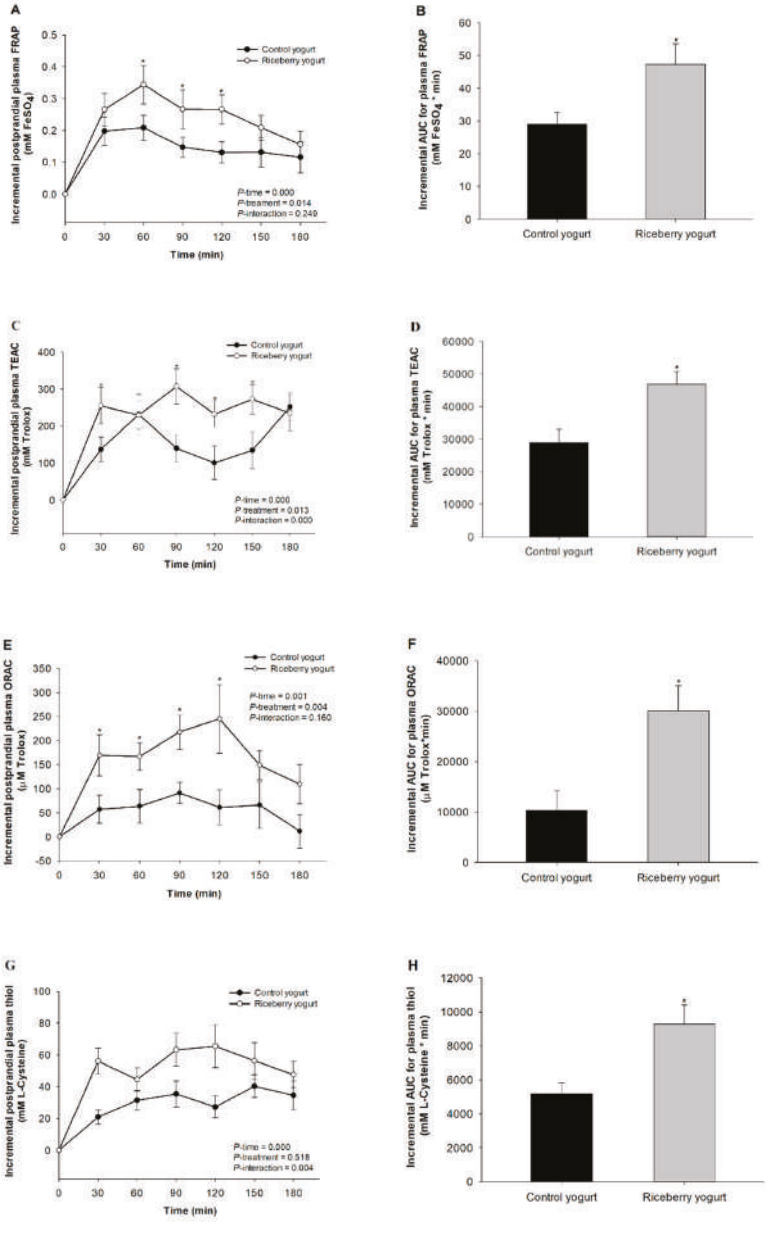

Figure 3. *Cont.*

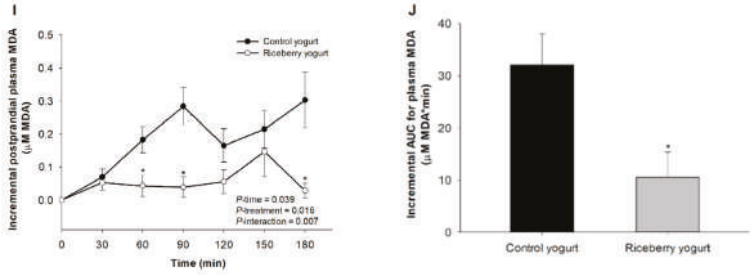

Figure 3. Incremental changes in postprandial plasma: ferric reducing ability of plasma (FRAP) (**A**); Trolox equivalent antioxidant capacity (TEAC) (**C**); oxygen radical absorbance capacity (ORAC) (**E**); thiol (**G**); and malondialdehyde concentration (MDA) (**I**). The incremental area under the curve (iAUC) for postprandial plasma: FRAP (**B**); TEAC (**D**); ORAC (**F**); thiol (**H**); and MDA (**J**) in healthy participants after consuming either the control yogurt (●) or riceberry rice yogurt (○). Data are presented as mean ± SEM, $n = 19$. * $p < 0.05$ compared to the control yogurt.

3.4. Subjective Rating

The subjective rating scores of hunger, fullness, desire to eat, and satiety after consumption of yogurts are illustrated in Figure 4A–D. All yogurts markedly reduced the score of hunger and desire to eat and increased the score of satiety and fullness after 30 min of ingestion as compared to baseline. However, there were no statistically significant differences in the rating score of hunger, fullness, desire to eat, and satiety among all yogurts.

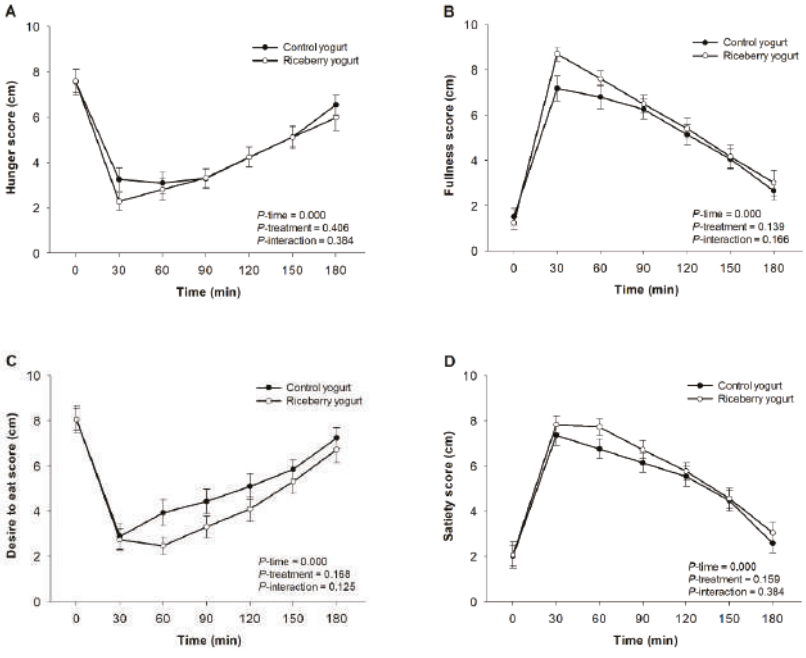

Figure 4. Incremental changes in subjective ratings for: hunger (**A**); fullness (**B**); desire to eat (**C**); and satiety (**D**) in healthy participants after consuming either the control yogurt (●) or riceberry rice yogurt (○). Data are presented as mean ± SEM, $n = 19$.

4. Discussion

Yogurt has received considerable attention as a potential approach to reduce the risks of weight gain, obesity, type 2 diabetes, and cardiovascular diseases [5,6]. Especially, several studies have reported the successful fortification of yogurt with bioactive compounds from edible plants such as green tea, black tea, white tea [21], chamomile [22], strawberry pulp [23], and aronia juice [10]. In our previous study, the supplementation of probiotic yogurt with anthocyanins from riceberry rice provided bioactive compounds and increased its functionality by increasing total phenolic content (TPC), cyanidin-3-glucoside (C3G), and peonidin-3-glucoside (P3G) concomitant with the elevation of DPPH radical scavenging activity and ferric reducing antioxidant power. In gastrointestinal digestion, this yogurt produced higher release of TPC and FRAP than the control [16]. Therefore, this was the first human study to investigate whether riceberry rice yogurt decreases postprandial glycemic response and improves antioxidant capacity in healthy volunteers. A reduction in the postprandial glucose excursion (40.23%) after consumption of riceberry rice yogurt was observed in healthy subjects. Cross-over studies have explored acute effects riceberry rice bread (50 g) on postprandial glycemic and insulin response in healthy volunteers [15]. After the riceberry rice bread intake, the AUC was 60% lower in comparison to jasmine rice bread. Furthermore, it was found to produce an attenuated postprandial insulin concentration after 15 min of consumption. The main reason to explain these effects is the ability of riceberry rice and its phytochemical compounds to inhibit carbohydrate digestive enzymes [15,24,25]. A previous study supports this reason that the anthocyanin-rich extract of riceberry rice was capable of inhibiting intestinal α-glucosidase such as maltase and sucrase [14]. Especially, C3G and P3G, the major anthocyanins identified in riceberry rice extract, was proved to be effective pancreatic α-amylase and α-glucosidase inhibitors [14,26,27]. Since lactose from milk was the major source of carbohydrate in the yogurt, a further study is needed to investigate the inhibitory effect of anthocyanins on lactase activity.

Postmeal hyperglycemia and glycemic fluctuations induces excessive production of reactive oxygen species (ROS). The excessive formation of ROS may be a contributing factor for induction of pathological changes related to the development of cardiovascular diseases [28]. Interestingly, dietary antioxidants help scavenge and neutralize excessive and inappropriate ROS, sequencing to balance against oxidant condition [29]. Scientific evidence suggests that consumption of phytochemical-rich plants improved plasma antioxidant capacity and reduced lipid peroxidation in humans [30–32]. The different assays have been commonly used to measure plasma antioxidant capacity such as ORAC, TEAC, and FRAP. The ORAC assay refers the ability of antioxidant molecules to inhibit peroxyl radical induced oxidation [33], whereas the TEAC assay indicates the ability of hydrogen-donating antioxidants to neutralize a radical cation in both lipophilic and hydrophilic environments [34]. FRAP assay could reflect the ability of antioxidants to reduce the reaction of Fe3+/Fe2+ couple. In addition, protein thiols in plasma has multifaceted functions, including a pivotal role in antioxidant defense [35]. Compared to fasting state, consumption of yogurt control slightly increased plasma antioxidant capacity (FRAP, TEAC, and ORAC) and protein thiol level. This result is entirely due to yogurt containing milk proteins and natural substances which have antioxidant activity [36]. Remarkably, plasma FRAP, TEAC, ORAC, and protein thiol levels were high 180 min after riceberry rice yogurt consumption, in comparison with the yogurt control. Increases in postprandial antioxidant capacity are supported by in vitro studies indicating that yogurt supplemented with riceberry rice extract (0.25%) had 4.8-fold higher FRAP than the control yogurt [16]. Similar results were also reported by Chusak et al. who found a greater increase in plasma FRAP following riceberry rice bread consumption [15]. A notable result from our study was a marked increase in plasma MDA above baseline after an intake of yogurt control, indicating that diet could induce postprandial lipid oxidation. This alteration was noticeably attenuated by riceberry rice yogurt consumption. These findings led us to hypothesize that riceberry rice yogurt would decrease postprandial oxidative stress related to their antioxidant activity.

Other clinical studies have also shown improvement in plasma antioxidant capacity together with reduction of lipid peroxidation following consumption of anthocyanin-rich plants such as butterfly

pea flower [37], Chilean berry [38], and açai berry [31]. It is suggested that the improvement of plasma antioxidant status may be partly attributed to the antioxidant activity of phenolic compounds [37]. In this context, C3G and P3G, the incorporated active ingredients in riceberry rice yogurt, have been recognized as antioxidant agents as represented by FRAP, TEAC, and ORAC assays [39,40]. In addition, C3G and P3G had the ability to reduce the formation of lipid peroxidation in UVB irradiation model and vitamin E-depleted rat [41,42]. Interestingly, after consumption of anthocyanin-rich strawberries and chokeberries, mainly C3G reduced plasma MDA concentration by 31% and 46%, respectively [43,44]. Through these actions, C3G and P3G in riceberry rice yogurt may play a role in an increase in plasma antioxidant capacity, leading to decrease in lipid peroxidation. However, other phytochemical compounds in riceberry rice may influence postprandial antioxidant capacity. Therefore, the quantification of the postprandial concentration of individual polyphenol after riceberry yogurt consumption is needed, which may help explore the role of riceberry rice yogurt in suppressing postprandial oxidative stress.

Visual analog scales (VAS) are relievable tools for the evaluation of subjective appetite sensation about hunger, fullness, desire to eat, and satiety [45]. In our study, the scores of all parameters did not show any significant differences between riceberry rice yogurt and the control yogurt. This finding is in agreement with the earlier study that bread made from riceberry rice did not alter subjective rating scores of hunger, fullness, desire to eat, and satiety in healthy adults. In addition, no change in postprandial level of glucagon-like peptide-1 (GLP-1), an incretin hormone, was detected following consumption of riceberry rice bread [15]. GLP-1 is responsible for the stimulation of insulin secretion, inhibition of glucagon secretion and gastric emptying, and regulation of appetite and satiety [46]. Interestingly, anthocyanins stimulated the secretion of GLP-1 from Murine GLUTag cell line [47]. We hypothesized that consumption of riceberry rice yogurt could not modulate satiety and appetite through the stimulation of GLP-1, possibly as a result from a small amount of anthocyanin in yogurt and its low bioavailability. Furthermore, we acknowledge some potential limitations to the current study. First, we did not introduce a full meal to consume with riceberry yogurt. Other macro- and micronutrients may interfere the postprandial effect of riceberry rice yogurt on plasma glucose and antioxidant capacity. Moreover, this study was only a relatively young and healthy population; older age subjects were not included to increase the homogeneity of postprandial response.

5. Conclusions

The present findings indicate that consumption of riceberry rice yogurt had a favorable effect in reducing postprandial plasma glucose and plasma MDA with improvement of plasma antioxidant status. With respect to appetite ratings, no significant change in scores of fullness, desire to eat, and satiety was observed following consumption of riceberry yogurt. The results suggest that riceberry rice yogurt could be a healthy food for improving the postprandial glycemic and antioxidant response in humans. The further study should determine the long-term effect of riceberry rice yogurt consumption in other populations at risk for chronic diseases following meals.

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Anthocyanin-Enriched Riceberry Rice Extract Inhibits Cell Proliferation and Adipogenesis in 3T3-L1 Preadipocytes by Downregulating Adipogenic Transcription Factors and Their Targeting Genes

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Abstract: Riceberry rice (*Oryza sativa* L.) is a new pigmented variety of rice from Thailand. Despite its high anthocyanin content, its effect on adipogenesis and adipocyte function remains unexplored. We investigated whether Riceberry rice extract (RBE) impacted cell proliferation by examining viability and cell cycle, using preadipocyte 3T3-L1 cells. To test RBE's effect on adipocyte formation, cells were cultured in adipogenic medium supplemented with extract and adipocyte number and triglyceride levels were quantified. Furthermore, Akt1 phosphorylation along with RT-qPCR and intracellular calcium imaging were performed to obtain an insight into its mechanism of action. The effect of RBE on adipocyte function was investigated using glucose uptake and lipolysis assays. Treatment of cells with RBE decreased preadipocyte number without cytotoxicity despite inducing cell cycle arrest ($p < 0.05$). During adipogenic differentiation, RBE supplementation reduced adipocyte number and triglyceride accumulation by downregulating transcription factors (e.g., PPARγ, C/EBPα, and C/EBPβ) and their target genes ($p < 0.05$). The Akt1 phosphorylation was decreased by RBE but insignificance, however, the extract failed to increase intracellular calcium signals. Finally, the treatment of adipocytes with RBE reduced glucose uptake by downregulating Glut4 mRNA expression and enhanced isoproterenol-induced lipolysis ($p < 0.05$). These findings suggest that RBE could potentially be used in the treatment of obesity by inhibiting adipocyte formation and proliferation.

Keywords: Riceberry rice; anthocyanin; cell proliferation; adipogenesis; preadipocytes; obesity; 3T3-L1 cells

1. Introduction

Obesity is an abnormal condition in which an imbalance between energy intake and energy expenditure occurs, leading to fat accumulation in adipose tissue [1]. It becomes life-threatening and reduces quality of life, as it increases the risk of developing non-communicable diseases (NCDs). In general, obesity is characterized by increased expansion of white adipose tissue, resulting

from increased fat-cell size (hypertrophy) and fat-cell number (hyperplasia or adipogenesis) [2]. Adipogenesis rapidly begins with the induction of initiating transcription factor, C/EBPβ mediated by 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone [3]. Then, C/EBPβ activates key adipogenic transcription factors including C/EBPα and PPARγ. Insulin is an important factor for the induction and maintenance of adipocytes by directly activating PPARγ and C/EBPα expression through the PI3K-Akt1 signaling pathway [3,4]. Consequently, PPARγ and C/EBPα contribute to the expression of their targeting adipogenic genes, resulting in increased cell differentiation, fatty acid transportation, glucose uptake, and lipogenesis [3]. In particular, the activation of the lipogenesis pathway increases excessive synthesis and accumulation of triglyceride in lipid droplets of mature adipocytes, leading to hypertrophy or increasing fat-cell size [5]. Studies revealed that hypertrophic obesity and adipose cell size are causes of insulin resistance [2].

Experiments to provide insight into the molecular mechanisms controlling adipogenesis of human adipocytes revealed that intracellular calcium signaling inhibits the early phase of adipogenesis but stimulates the later phase and causes lipid filling [6]. Currently, attempts are being made to identify naturally occurring bioactive compounds that can suppress preadipocyte proliferation and adipogenesis [7].

Anthocyanins, a group of water-soluble polyphenolic compounds, are responsible for the red, purple, and blue color in fruits, vegetables, and plants. Anthocyanins have many health benefits such as antioxidant, anti-cancer, preventing cardiovascular disease, neuroprotective effect, anti-diabetes, and anti-obesity effects [8]. Studies show that anthocyanins possess anti-obesity activity through the regulation of adipocytes, including induction of cell cycle arrest, reduction of lipid accumulation by suppressing transcription factors expressions such as PPARγ, C/EBPα, C/EBPβ, C/EBPδ, and aP2, and promoting lipolysis [9]. Interestingly, anthocyanin-enriched extracts from fermented blueberry juice, cranberry, blue pea flower (*Clitoria ternatea*), and blueberry peel inhibits adipogenesis and lipogenesis in 3T3-L1 adipocytes via downregulation of adipogenic genes [10–14].

Riceberry rice (*Oryza sativa* L.), is a dark-purple rice originated from Hom Nin rice and Hom Mali 105 rice. The pigment from this rice contains anthocyanins which are cyanidin-3-*O*-glucoside (C3G) and peonidin-3-*O*-glucoside (P3G) [15,16]. An extract from Riceberry rice bran has a cytoprotective effect from oxidative damage [17], and prevents nephrotoxicity [18], as well as hepatotoxicity [19] in rats. In addition, it inhibits cancer cell proliferation and promotes apoptosis by inducing cell cycle arrest and DNA fragmentation, increasing p53 protein expression, decreasing caspase-3 protein expression [15,20]. Interestingly, Riceberry rice bran oil improves glycemic levels in diabetic rats by increasing the expression of glucose transporter 4 (Glut4) in muscle [16,21]. Studies with Riceberry rice extract (RBE) revealed that it inhibits key enzymes and steps of carbohydrate and lipid digestion and absorption [22]. Consumption of Riceberry rice bread reduces glycemic responses together with the improvement of antioxidant status in healthy subjects [23,24]. However, there is very limited information regarding the anti-obesity effect of anthocyanin-enriched extract from RBE.

The objective of this study is to investigate the effect of RBE on preadipocyte proliferation and adipogenesis in 3T3-L1 cells. We determined whether intracellular calcium signaling was involved in the RBE responses and examined its impact on transcription factors, adipogenic gene expression, and adipocyte function.

2. Materials and Methods

2.1. Materials

Mouse 3T3-L1 preadipocytes (CL-173™) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MUSE™ Cell Count and Viability and MUSE™ Cell Cycle kits were purchased from Merck (Millipore, Darmstadt, Germany). Triglyceride liquicolor GPO-POD kit was purchased from Human® (Human, Wiesbaden, Germany). All gene-specific mouse primers

were generated from IDT™ (Integrated DNA Technologies, Inc., Coralville, IA, USA). The TRIzol™, 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-amino)-2-Deoxyglucose (2-NBDG) and Dulbecco's Modified Eagles Medium (DMEM)/high glucose were purchased from Invitrogen™ (Thermo Fisher, Waltham, MA, USA). The RQ1 DNase kit and reverse transcription system were purchased from Promega (Promega®, Madison, WI, USA). An iTaq™ Universal SYBR® Green Supermix was purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA).

2.2. Extraction of Riceberry Rice and Phytochemical Analysis

Riceberry rice harvested in Thailand was purchased from the local market. Briefly, the rice (2 kg) was extracted in 5 L of water at 50 °C for 40 min, then followed by freeze dryer lyophilization. Riceberry rice extract (RBE) was dissolved in distilled water (2 mg/mL) before use. Total phenolic content in RBE was determined using the Folin-Ciocalteau method with minor modification [25]. An aliquot of RBE (50 µL) was incubated with 50 µL of Folin-Ciocalteau reagent. After 5 min incubation in the dark, the mixture was incubated with 50 µL of 10% (*w*/*v*) sodium carbonate for 30 min. The absorbance of the mixture was measured at 760 nm. The total phenolic content was expressed as mg of gallic acid equivalents per g of extract (mg GAE/g extract). Total flavonoid content in RBE was determined as previously described [26]. Then, 100 µL of RBE was mixed with 30 µL of 5% (*w*/*v*) sodium nitrite and 400 µL of water. After 5 min incubation in the dark, 30 µL of 10% (*w*/*v*) aluminum chloride, 200 µL of 1 M sodium hydroxide, and 240 µL of water were added to each mixture. The absorbance was measured at 510 nm using a spectrophotometer. Total flavonoid content was expressed as mg of catechin equivalents per g of extract (mg CE/g extract). Quantification of total anthocyanin content in the extract was determined using a pH differential method [27]. RBE (500 µL) was incubated with 500 µL of two different buffer solutions including 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5) for 15 min in the dark. The absorbance of each mixture was measured at 520 and 700 nm then calculated using the equation of A = $(A520–A700)_{pH1.0}$–$(A520–A700)_{pH4.5}$. The total anthocyanin content was expressed as mg of cyanidin-3-glucoside per g of extract (mg C3G/g extract).

2.3. HPLC

To quantify the major anthocyanins in RBE, high-performance liquid chromatography (HPLC) was performed using a C18 column (250 × 4.6 mm, 5 µm, Varian®) with minor modifications [28]. The extract (1 mg/mL) was dissolved in methanol with 2% (*v*/*v*) HCl solution. The absorbance was detected at 515 nm. Cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) were used as standards. The results were expressed as µg of C3G or P3G per mg of extract.

2.4. UHPLC-ESI-Q-TOF-MS/MS

To characterize the phytochemical contents in RBE, liquid chromatography and tandem mass spectrometry (LC-MS/MS) were performed using a Titan C18 reverse phase column (50 × 21 mm, 1.9 µm particle size). The RBE was dissolved in a 0.1% formic acid solution to a final concentration of 10 mg/mL and filtered through a 0.22 µm Nylon syringe filter before injecting into the system at 0.3 mL/min flow rate for 20 min. The LC-MS/MS was carried out using an Ultimate 3000 UHPLC system (Thermo Scientific, Dionex, Sunnyvale, CA, USA) equipped with an Electrospray ionization-Quadrupole-Time of Flight Mass Spectrometer (ESI-Q-TOF-MS/MS; Model Impact II, Bruker Daltonik GmbH, Bremen, Germany). The HPLC gradients which included eluent A; 0.1% formic acid in water and eluent B; 0.1% formic acid in acetonitrile was run with the multistep linear gradient; 0–9 min: 5–30% B; 12–17 min: 95% B; 17.5 min: 5% B and held for 2 min. The column temperature was maintained at 30 °C and the injection volume was 5 and 10 µL for positive, and negative ionization mode, respectively.

The mass spectra were recorded under the following ESI inlet conditions: the capillary voltage of 3800 V for positive mode and 2500 V for negative mode, the scanning mass-to-charge (*m*/*z*) range of 50 to 1000, the pressure of the nebulizer at 2.0 bar, the drying gas temperature at 200 °C, and drying gas flow, 8.0 L/min. Automatic MS/MS experiments were performed adjusting the collision energy values

20–50 eV depending on *m*/*z* and using nitrogen as collision gas. Sodium formate solution was used as a calibrant for auto internal mass calibration.

The MS data were processed through Data Analysis 4.3 software (Bruker Daltonics, Bermen, Germany). The identification was performed by using MS-DIAL software (RIKEN, version 4.18) which matching experimental mass spectra against mass spectral libraries based on weighted similarity score of accurate mass and MS/MS spectra. Respect and GNPS mass spectral libraries were used and a cut off value of 80% was selected.

2.5. Cell Culture

For proliferation assay, mouse 3T3-L1 preadipocytes were cultured in Dulbecco's Modified Eagles Medium (DMEM)/high glucose (HG) with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 5% CO_2 at 37 °C. Cells were seeded on 12-well plate (10,000 cells/mL) and cultured for 24 h followed by incubation with 1, 10, and 20 µg/mL RBE for 4 days. At this day, cells (control group) were grown in monolayer at 100% confluency. Cell viability, total cell number, and cell cycle were determined using MUSE™ Cell Count and Viability kit and MUSE™ Cell Cycle kit (Figure 1).

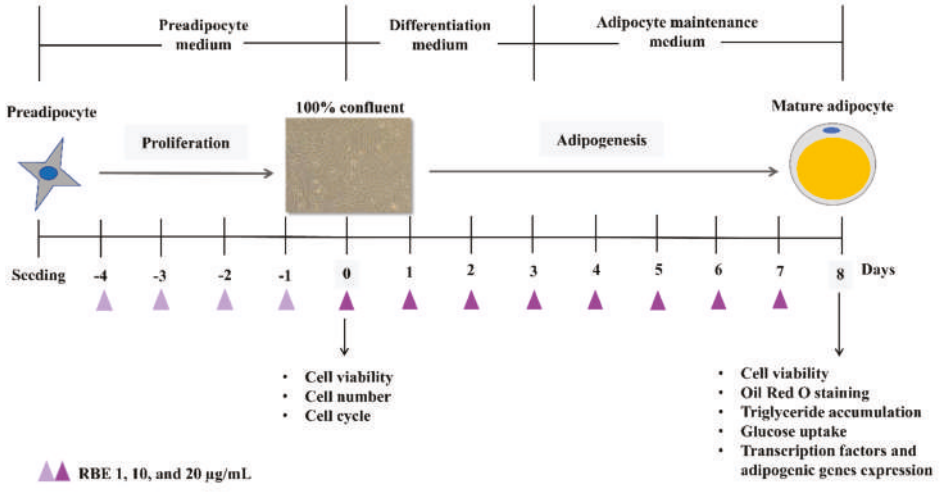

Figure 1. Schematic representation of 3T3-L1 differentiation into adipocytes.

For cell differentiation, preadipocytes were seeded on 12 well plates (10,000 cells/mL) and cultured in DMEM/HG with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin at 5% CO_2 at 37 °C. According to the proliferation experiment, cells were cultured for 4 days until 100% confluent. In early phase of adipogenesis, cells were induced by medium supplemented with 0.5 mM 3-isobutryl-1-methylxanthine (IBMX), 1 µM dexamethasone, and 2.5 µg/mL insulin for 3 days. In late phase of adipogenesis, cells were maintained in culture medium supplemented with 2.5 µg/mL insulin and incubated with 1, 10, and 20 µg/mL RBE until fully differentiation on day 8 (Figure 1). Cell viability, mature adipocyte number (Oil Red O staining), triglyceride levels, the glucose uptake activity, and mRNA expression of major transcription factors and adipogenic genes were then examined. All experiments were performed with 3T3-L1 cells from passages 4–16.

2.6. Cell Number and Viability

After 4 days of treatment, cells were trypsinized and resuspended in DMEM. A 20 μL of suspended cells were incubated with 380 μL of MUSE™ Cell Count and Viability reagent for 5 min in the dark. The mixture was loaded into the MUSE™ Cell Analyzer (Millipore) to quantify the number of viable and dead cells in each sample. The results were expressed as a percentage of cell viability (% of control) and total cell number (cells/mL).

After RBE treatment for 8 days, cells were trypsinized and resuspended in 1 mL DMEM and mixed with trypan blue in a 1:1 ratio and counted using a hemocytometer. The results were expressed as a percentage of cell viability (% of control) and total cell number (cells/mL).

2.7. Cell Cycle

After 4 days of treatment, the cell cycle of preadipocytes was determined using MUSE™ Cell Cycle kit (Millipore, Germany). Cells were trypsinized and resuspended in 1 mL PBS (pH 7.4) and fixed with 3 mL of 70% cooled ethanol at −20 °C overnight. After that, fixed cells were washed and resuspended two times in PBS. Then, the cell pellets were resuspended in 200 μL of MUSE™ Cell Cycle reagent and incubated for 30 min in the dark. Finally, the mixture was loaded into the MUSE™ Cell Analyzer (Millipore) to quantify the proportion of cells in each stage of the cell cycle. The results were expressed as the percentage of cells in G0/G1, S, and G2/M phase.

2.8. Real-Time Calcium Imaging Analysis

To obtain insight into the molecular mechanism of RBE, intracellular calcium recording was performed in 3T3-L1 cells [29]. Cells were cultured on round glass coverslips for 48 h until 90–100% confluent then incubated at 37 °C for 30 min with 2 μM Fura-2AM in calcium imaging buffer consisting of 136 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM HEPES, 4 mM glucose, and 0.1% BSA at pH 7.4. The calcium signals were recorded using a dual excitation fluorometric imaging system (TILL-Photonics Grafelfingen, Gräfelfing, Germany) with excitation wavelength at 340 nm and 380 nm controlled by TILLvisION software. Fluorescence emissions were collected at 1 Hz, computed as an F340/F380 ratio, and expressed as the peak of calcium signals from individual cells. Ionomycin (1 μM) was used as a positive control.

2.9. Oil Red O Staining

Mature adipocytes were washed twice in PBS and fixed with 10% formalin overnight. Cells were washed twice with water and incubated with 60% isopropanol for 5 min. Then, cells were stained with Oil Red O solution for 20 min and the excess dye was washed five times with water. The stained lipid droplets were captured by an inverted microscope and the number of adipocytes quantified using ImageJ software. The results were expressed as total adipocyte count (cells/frame).

2.10. Determination of Triglyceride Level

Mature adipocytes were washed with PBS and lysed under sonication for 5 min following the previous study with modifications [30]. After centrifugation at 12,000× *g* for 10 min, 5 μL of supernatant was incubated with 250 μL of triglyceride reagent for 10 min in the dark. The absorbance was measured at 500 nm. The results were expressed as mg of triglyceride per mL (mg/mL).

2.11. Glucose Uptake

Glucose uptake was performed according to the previous method with slight modification [31]. After 8 days of incubation, mature adipocytes were incubated in PBS at 37 °C for 2 h, then incubated with 80 μM of fluorescent glucose analogue (2-NBDG) and 100 nM insulin at 37 °C for 60 min. The excess 2-NBDG was washed three times with ice-cold PBS. The fluorescence intensity of 2-NBDG was measured at 485 nm excitation wavelength and 535 nm emission wavelength using a fluorescence

microplate reader. Data were normalized to the total protein concentration from the BCA kit (Thermo Fisher, USA) using BSA as a standard. The results were expressed as the percentage of glucose uptake (% of control).

2.12. Determination of mRNA Expression

At day 8 of differentiation, total RNA was extracted using TRIzol™ reagent (Invitrogen™, Thermo Fisher, USA). Quantification of RNA was determined using NanoDrop 1000 spectrophotometer. Total RNA (200 ng/μL) was treated with RQ1 DNase enzyme using RQ1 DNase treatment kit (Promega®, USA). After that, DNase-treated RNA was converted into cDNA using Reverse transcription system (Promega®, USA). Finally, 25 ng/μL of cDNA template was mixed with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA) and gene-specific mouse primers as shown in Table S1. RT-qPCR was carried out in a CFX384 TouchTM Real-Time PCR Detection system (Bio-RAD, CA, USA) using SYRB green detection according to the manufacturer's instruction. The mRNA expression was normalized with β-actin using the $2^{-\Delta\Delta Ct}$ method. The result was expressed as the relative mRNA expression.

2.13. Determination of Akt1

To investigate the effects of RBE on Akt1 signaling in adipocytes, 3T3-L1 cells were cultured and differentiated in 6-well plate. After treatment with RBE for 8 days, cells were washed with cold PBS and lysed with 200 μL/well of ice-cold 1X MILLIPLEX® MAP lysis buffer (EMD Millipore, Merck, Germany). Cell lysates were gently rocked for 15 min at 4 °C and centrifuged at 14,000× *g* under 4 °C for 15 min. Supernatants were collected and stored at −80 °C for further experiments. The phosphorylation levels of Akt1 (Ser473) were determined using the MILLIPLEX® MAP Phospho/Total Akt1 2-plex Magnetic Bead Panel kit (EMD Millipore, Merck, Germany) according to the manufacturer's instruction. The fluorescence intensity of the beads was measured and analyzed using the Luminex® system (EMD Millipore, Merck, Germany). Data were normalized to the total protein concentration from the BCA kit (Thermo Fisher, USA) using BSA as a standard. The results were expressed as the Median Fluorescence Intensity (MFI) per mg protein (MFI/mg protein).

2.14. Lipolysis

Glycerol release represented the lipolysis rate and determined according to published method with minor modifications [32]. Mature adipocytes were starved in serum-free DMEM at 37 °C overnight. To obtain a basal control level, cells were treated with RBE only for 3 h. For lipolysis stimulation, cells were supplemented for 3 h with RBE and 100 nM isoproterenol. The media in each sample was incubated with free glycerol reagent (Sigma Aldrich, Germany) at 37 °C for 5 min. The absorbance was measured at 540 nm. The glycerol levels were calculated using a calibration curve of glycerol standard (0–260 μL/mL). Data were normalized to the total protein concentration from the BCA kit (Thermo Fisher, USA) using BSA as a standard. The results were expressed as mM of glycerol per mg protein (mM/mg protein).

2.15. Statistical Analysis

Data were expressed as mean ± standard error of the mean (SEM) from three independent experiments ($n = 3$). The statistical significance was analyzed using One-way analysis of variance (ANOVA) with Duncan's post hoc test using SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was established at $p < 0.05$.

3. Results

3.1. Phytochemical Composition in RBE

Total phenolic compounds, flavonoids, and anthocyanins in RBE were 63.33 ± 2.40 mg GAE/g extract, 18.00 ± 0.01 mg CE/g extract, and 10.13 ± 0.14 mg C3G/g extract, respectively. The detected

anthocyanins were cyanidin-3-glucoside (2.05 ± 0.04 μg/mg extract) and peonidin-3-glucoside (0.78 ± 0.01 μg/mg extract). As reported in the previous study, RBE from acidic methanol with solid-phase extraction contained higher amount of total phenolic compounds, flavonoids, and anthocyanins than the obtained results from current study [22]. It may be because of different extracting solvents and methods. According to the chromatograms from UHPLC-MS/MS (Figure S1), seven compounds were identified based on their retention times, high-resolution mass spectra of the fragment ions, and compared to the previous study, including cyanidin-3-glucoside (C3G), peonidin-3-glucoside (P3G), petunidin-3-glucoside, caffeic acid, taxifolin, quercetin-3-rutinoside or rutin, and ferulic acid (Tables S2 and S3) [33].

3.2. RBE Inhibited Cell Proliferation of Preadipocytes

RBE (1–20 μg/mL) did not affect cell viability after 4 days of treatment (Figure 2A). The results also found that RBE at 20 μg/mL significantly reduced preadipocyte total cell number by 49% (Figure 2B). In addition, RBE at 10 and 20 μg/mL increased the proportion of cells at the G0/G1 phase with a concomitant decrease in the G2/M phase (Figure 2C).

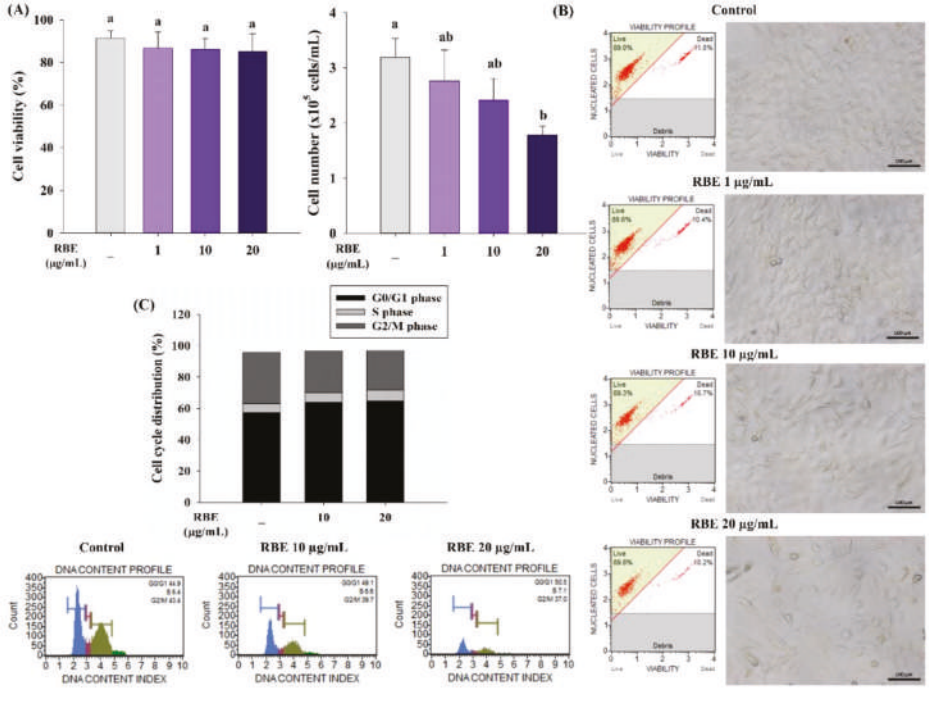

Figure 2. Effect of Riceberry rice extract (RBE) on 3T3-L1 preadipocyte proliferation during a 4-day period. (**A**) Treatment of cells with RBE did not affect cell viability. (**B**) RBE at 20 μg/mL significantly decreased the total cell number. Bright-field images (20× magnification) show the reduction in preadipocyte under RBE treatment compared to the control group. (**C**) RBE at 10 and 20 μg/mL induced cell cycle arrest with increased G0/G1 and decreased G2/M phases. The results are expressed as mean ± SEM from the three independent experiments. $p < 0.05$ for groups with different letters. Scale bars are 100 μm.

3.3. Effect of RBE on Intracellular Calcium Signaling in Preadipocytes

Since intracellular calcium signaling is known to control adipogenesis, we examined whether RBE utilized this signaling mechanism to exert its effect in preadipocytes. Stimulation of cells with RBE (1–20 μg/mL) failed to increase intracellular calcium signals compared to ionomycin treatment (positive control) (Figure 3A). The increases in cell fluorescence during RBE and ionomycin stimulation are shown in Figure 3B.

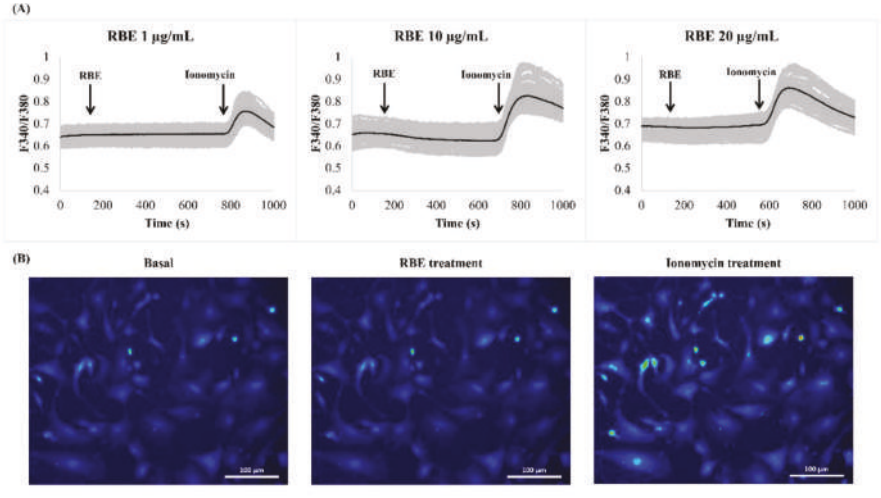

Figure 3. Effect of Riceberry rice extract (RBE) on intracellular calcium signals in 3T3-L1 cells. (**A**) Stimulation of cells with RBE 1, 10, and 20 μg/mL concentrations failed to induce calcium signals compared to 1 μM ionomycin as a positive control. Black lines represent average traces from all cells. Grey lines represent individual cell recordings. (**B**) Fluorescence emission from cells prior to, during RBE, and ionomycin treatments. Scale bars are 100 μm.

3.4. Effect of RBE on Adipogenesis

In order to examine RBE's effect on adipogenesis, preadipocytes were differentiated in adipogenic medium supplemented with RBE for 8 days. Treatment of cells with RBE significantly reduced the number of adipocytes in a concentration-dependent manner without affecting cell viability (Figure 4A,C). Moreover, RBE (20 μg/mL) significantly reduced the total adipocyte number by 23% (Figure 4B). In comparison with the control group, the level of triglyceride accumulation in adipocytes was significantly lowered by RBE (1–20 μg/mL) (Figure 4D). The morphological changes of stained adipocytes after exposure to adipogenic medium with RBE are shown in Figure 4E. Mature adipocytes demonstrated round-shape with lipid droplets in the cytoplasm compared to undifferentiated cells.

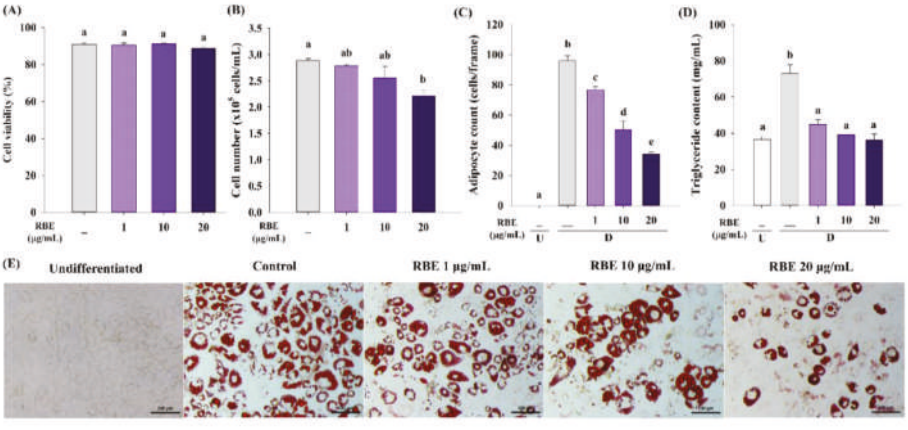

Figure 4. Effect of Riceberry rice extract (RBE) on adipogenesis in 3T3-L1 cells during an 8-day period. (**A**) Treatment of cells with RBE did not affect cell viability during adipogenesis. (**B**) RBE at 20 μg/mL significantly decreased the total adipocyte number. (**C**) RBE (1–20 μg/mL) decreased adipocyte number in a concentration-dependent manner. (**D**) A significant reduction in triglyceride accumulation was observed in mature adipocytes. (**E**) Treatment of cells with RBE inhibited adipogenesis by decreasing adipocyte number, cell size, and lipid droplet content as shown by Oil Red O staining (20× magnification). The results are expressed as mean ± SEM from the three independent experiments. Groups with a different letter represent statistical significance ($p < 0.05$). U: undifferentiated cells; D: differentiated cells. Scale bars are 100 μm.

3.5. Effect of RBE on Transcription Factors and Akt1 Signaling in Late Phase of Adipogenesis

After 8 days of adipogenic differentiation, the mRNA expression levels of PPARγ, C/EBPα, and C/EBPβ significantly increased in differentiated cells compared to undifferentiated control. Interestingly, RBE (20 μg/mL) significantly downregulated PPARγ, C/EBPα, and C/EBPβ mRNA expression but upregulated C/EBPγ mRNA expression (Figure 5A). In addition, differentiated cells demonstrated an increase in the relative value of p-Akt1 (Ser473)/total-Akt1, whereas RBE (20 μg/mL) tended to decrease its relative value (Figure 5B).

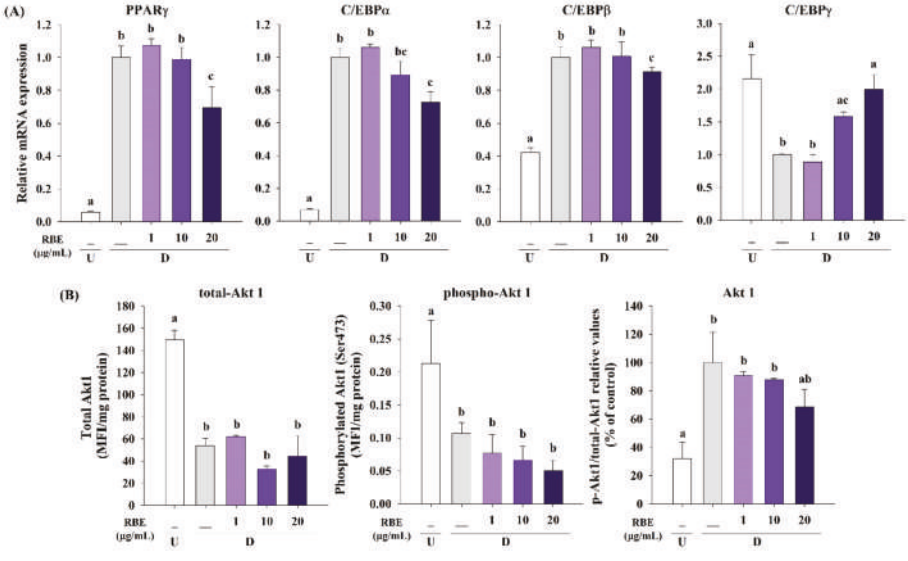

Figure 5. Effect of Riceberry rice extract (RBE) on adipogenic transcription factors and Akt1 signaling in 3T3-L1 cells. (**A**) RBE (20 μg/mL) significantly downregulated mRNA expression of PPARγ, C/EBPα, and C/EBPβ, and upregulated C/EBPγ in differentiated cells. (**B**) Treatment of cells with RBE tended to reduce the relative values of phospho-Akt1 (S473) to total-Akt1 in differentiated cells. The results are expressed as mean ± SEM from the three independent experiments. Groups with a different letter represent statistical significance ($p < 0.05$). Data were normalized with β-actin as an internal control. U: undifferentiated cells; D: differentiated cells.

3.6. Effect of RBE on Adipogenic Gene Expression

Based on the inhibitory effects of RBE on transcription factors and Akt1 signaling, its impact on adipogenic gene expression was determined. Treatment of cells with RBE (20 μg/mL) significantly downregulated the expression of adipogenic genes ACC, aP2, AdipoQ, leptin, resistin, perilipin, HSL, LPL, ATGL, and adiponectin receptors R1 and R2 (AdipoQ-R1 and AdipoQ-R2) compared to control differentiated cells in the absence of RBE (Figure 6).

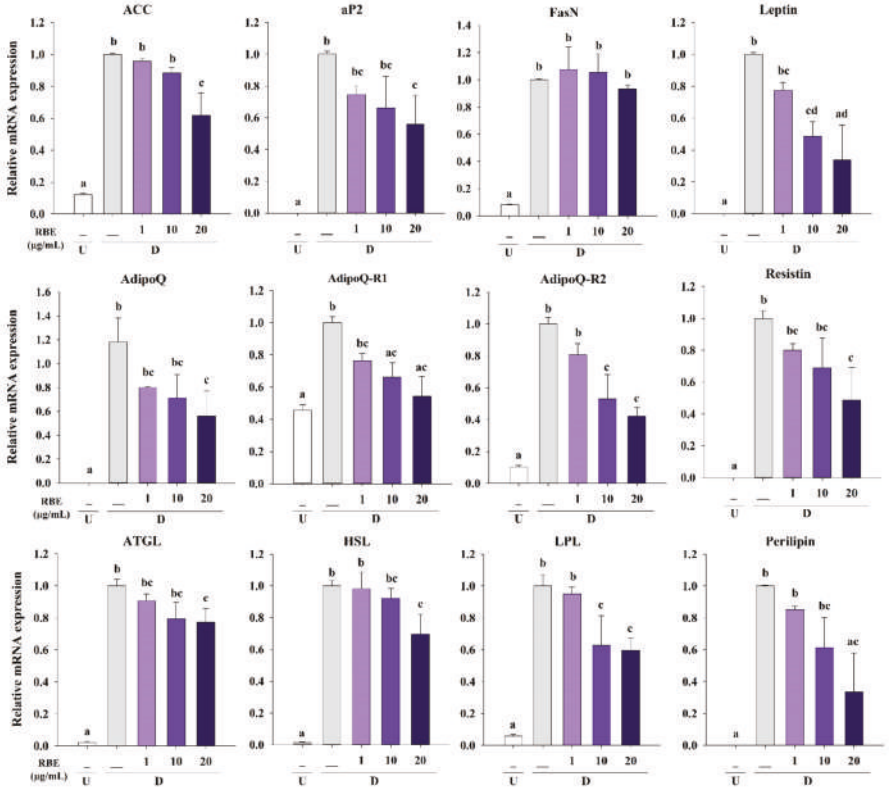

Figure 6. Effect of Riceberry rice extract (RBE) on adipogenic gene mRNA expression. Treatment with RBE (20 μg/mL) significantly downregulated adipogenic gene mRNA expression for ACC, aP2, Leptin, AdipoQ, AdipoQ-R1, AdipoQ-R2, Resistin, ATGL, HSL, LPL, and perilipin in differentiated cells. The results are expressed as mean ± SEM from the three independent experiments. Groups with a different letter represent statistical significance ($p < 0.05$). Data were normalized with β-actin as an internal control. U: undifferentiated cells; D: differentiated cells.

3.7. Effect of RBE on Glucose Uptake, the Glut4 mRNA Expression, and Lipolysis

We examined whether RBE impacted glucose uptake in adipocytes and Glut4 gene expression. The results revealed that RBE at 10 and 20 μg/mL caused a 16% inhibition in glucose uptake compared to control untreated cells (Figure 7A). Moreover, RBE (20 μg/mL) treatment reduced Glut4 mRNA expression (Figure 7B) without affecting insulin receptor expression (Figure 7C). The experiments testing the effect of RBE on lipolysis revealed that it tended to increase the basal lipolysis, whereas significantly enhanced isoproterenol-induced glycerol release by 35%, compared to the control cells in the absence of RBE (20 μg/mL) (Figure 8).

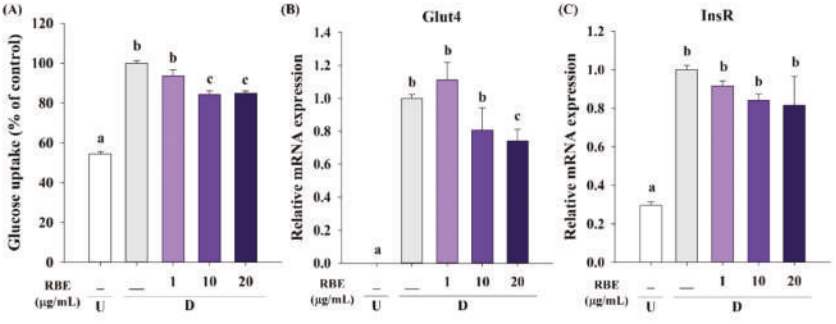

Figure 7. Effect of Riceberry rice extract (RBE) on glucose uptake in mature adipocytes. (**A**) RBE at 10 and 20 μg/mL significantly reduced glucose uptake into mature adipocytes after 8 days of treatment. (**B**) Downregulation of Glut4 mRNA expression in response to RBE at 20 μg/mL in differentiated cells. (**C**) Treatment of cells with RBE did not impact insulin receptor (InsR) mRNA expression. The results are expressed as mean ± SEM from the three independent experiments. Groups with a different letter represent statistical significance ($p < 0.05$). Data were normalized with β-actin as an internal control. U: undifferentiated cells; D: differentiated cells.

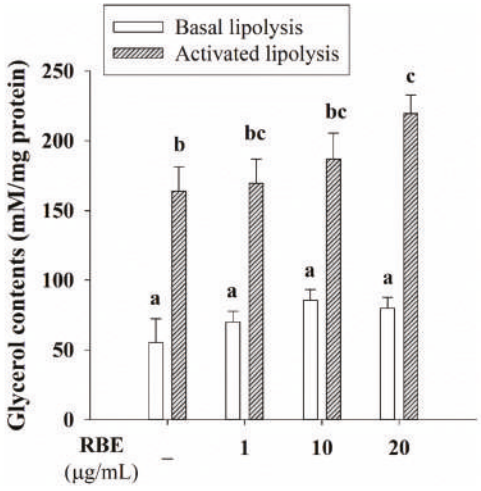

Figure 8. Effect of Riceberry rice extract (RBE) on lipolysis in adipocytes. RBE (20 μg/mL) significantly enhanced isoproterenol-induced glycerol release from adipocytes. The results are expressed as mean ± SEM from the three independent experiments. Groups with a different letter represent statistical significance ($p < 0.05$).

4. Discussion

One of the most promising mechanisms by which natural products counteract obesity is by inhibiting adipogenesis, blocking preadipocyte proliferation and/or differentiation [2]. Aguilar et al. revealed that the inhibition of cell proliferation, by inducing the cell cycle arrest, can control adipogenesis [34]. In the present study, RBE demonstrated an anti-proliferative effect by reducing the number of viable preadipocytes without compromising cell viability. The results from flow cytometry assay indicated that treatment of 3T3-L1 preadipocytes with RBE inhibited cell proliferation by inducing cell cycle arrest at the G0/G1 and G2/M phases. During adipogenic differentiation, RBE also decreased

the cell population which could be attributed to its sustained anti-proliferative effect. These findings are consistent with reports showing an anti-proliferative effect on 3T3-L1 preadipocytes by black soybean anthocyanins during adipocyte differentiation [35]. Real-time calcium imaging experiments, addressing the molecular mechanism, by which RBE inhibited cell proliferation and adipogenesis, revealed that calcium signals are not involved in its mechanism of action. These findings differ from studies demonstrating the importance of calcium signals for the adipocyte cell cycle and the early stages of adipogenesis [6,36,37]. Therefore, the mechanism of RBE might be the calcium-independent mechanisms as reported in previous studies, such as coffee extract inhibits preadipocyte proliferation and differentiation by interrupting the insulin signaling pathway by decreasing protein expression of the insulin receptor substrate 1 (IRS1) and further promoting their degradation [38].

Fibroblast-like preadipocytes begin to differentiate into round-shape mature adipocytes when exposed to adipogenic medium containing IBMX, dexamethasone, and insulin [5]. This process involves a cascade of transcription factors. Initially, there is the upregulation of transcription factor C/EBPβ by IBMX and dexamethasone during the early stage of adipocyte differentiation [39]. This is followed by PPARγ and C/EBPα gene expression, the master regulators of adipogenesis. Another member of the C/EBP family, C/EBPγ inhibits adipogenesis through heterodimerization and inactivation of C/EBPβ [39]. Our results revealed that RBE exerted the inhibitory effect on PPARγ and C/EBPα expression at least in part by upregulation C/EBPγ and downregulation of C/EBPβ during adipogenic differentiation. The activation of the PI3K-Akt signaling pathway by insulin enhances the expression of PPARγ and C/EBPα during preadipocyte differentiation [3]. Interestingly, RBE did not inhibit Akt1 phosphorylation, suggesting that the anti-adipogenic effects may not be attributed to the insulin-mediated PI3K-Akt signaling pathway. One alternative mechanism could be the downregulation of C/EBPβ with inhibition of the MEK-ERK and Akt1 pathways, leading to decreases in PPARγ and C/EBPα expression [38].

Apart from PPARγ and C/EBPα, adipocyte differentiation involves the expression of several genes critical for the development of the adipose phenotype and biosynthesis, including the lipogenic pathway, adipokine secretion, and insulin sensitivity [3]. In mature adipocytes, triglyceride synthesis is regulated by increasing lipogenic genes expression such as perilipin (lipid droplet-associated protein), fatty acid-binding protein (FABP or aP2), acetyl-CoA carboxylase (ACC, a fatty acid-synthesis enzyme), fatty acid synthase (FasN), lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), and adipose triglyceride lipase (ATGL) [5,40]. In addition, it requires the expression of important adipokines genes (adiponectin, leptin, and resistin) [41] and glucose transporter 4 (Glut4) for glucose uptake into mature adipocytes [3]. Consistent with the Oil Red O staining and triglyceride accumulation experiments, RBE significantly decreased the expression of lipogenic genes including perilipin, aP2, ACC, LPL, HSL, and ATGL. Adipokine marker expression profiles (AdipoQ, AdipoQ-R1, AdipoQ-R2, leptin, and resistin) were also reduced by RBE treatment during adipogenesis. Furthermore, RBE suppressed Glut4 gene expression, leading to decrease glucose uptake in adipocytes. The suppression of adipogenic gene expression by RBE inhibited adipocyte proliferation and differentiation as well as downregulation of key transcription factors. These findings are consistent with studies where anthocyanin-rich plants, such as cranberry and blue pea flower extract, are capable of inhibiting adipogenesis by downregulating adipogenic transcription factors and their targets [11,12,14].

Lipolysis catalyzes the breakdown of triglyceride in intracellular lipid droplets, leading to reduce the fat-cell size and hypertrophic adipocytes [42]. Generally, there are two processes of lipolysis: basal lipolysis and activated lipolysis by catecholamine [42]. Isoproterenol or catecholamines bind to the β3-adrenergic receptor on adipocytes and promote lipolysis through the cAMP-PKA signaling pathway [42]. Our study revealed that RBE had a tendency to enhance catecholamine-stimulated lipolysis in 3T3-L1 cells that is consistent with anthocyanin-rich *Clitoria ternatea* flower petal extract [14]. This finding suggests a beneficial effect of RBE on catecholamine-induced lipolysis in mature adipocytes.

Several reports show that phytochemical compounds in plants can inhibit adipogenesis by downregulating adipogenic transcription factors in preadipocytes. In particular, black rice (*Oryza sativa* L.) extract inhibited adipogenesis by downregulating the mRNA expression of PPARγ, C/EBPα,

LPL, and aP2 in mesenchymal C3H10T1/2 cells [43]. Jang et al. reported that C3G and P3G present in black rice extract are promising anti-adipogenic compounds [43]. Apart from anthocyanins, phenolic compounds in RBE, including quercetin-3-rutinoside or rutin, caffeic acid, and ferulic acid could have similar action as adipogenic inhibitors [44]. Among these phenolic compounds, rutin expressed the highest inhibitory activity on lipid accumulation in 3T3-L1 cells followed by caffeic acid, and ferulic acid, respectively [44]. Rutin suppressed the adipocyte differentiation by downregulating the expression of PPARγ, C/EBPα, FasN, LPL, and aP2 [45,46]. Moreover, coffee extract which mainly contains caffeic acid showed the anti-adipogenic effects by suppressing the expression of adipocyte marker genes including PPARγ, C/EBPα, aP2, LPL, Glut4 and adiponectin [47]. Interestingly, ferulic acid binds to PPARγ leading to conformational changes and decreases PPARγ-target genes expression including aP2, FasN, LPL, perilipin1, and adiponectin [48]. The binding to PPARγ by phenolic compounds may be responsible for inhibition of adipocyte differentiation. Our findings suggest that anthocyanins (C3G and P3G) and phenolic compounds (rutin, caffeic acid, and ferulic acid) in RBE could be responsible for its anti-adipogenic effect.

5. Conclusions

This study suggested that anthocyanin-enriched RBE inhibited cell proliferation and differentiation in 3T3-L1 cells (Figure 9). In addition, RBE blocked the early stage of adipocyte proliferation by inducing cell cycle arrest at G0/G1 and G2/M phases. In the process of cell differentiation, RBE upregulated transcription factors C/EBPγ with concomitant downregulation of major transcription factors including C/EBPβ, PPARγ, and C/EBPα and their target genes such as ACC, aP2, LPL, HSL, ATGL, adiponectin, leptin, resistin, and perilipin. This resulted in decreased triglyceride accumulation in adipocytes. Moreover, RBE reduced glucose uptake by downregulating Glut4 mRNA expression and enhanced catecholamine-induced lipolysis in mature adipocytes. These findings suggest a potential application for RBE in the treatment of obesity and related diseases.

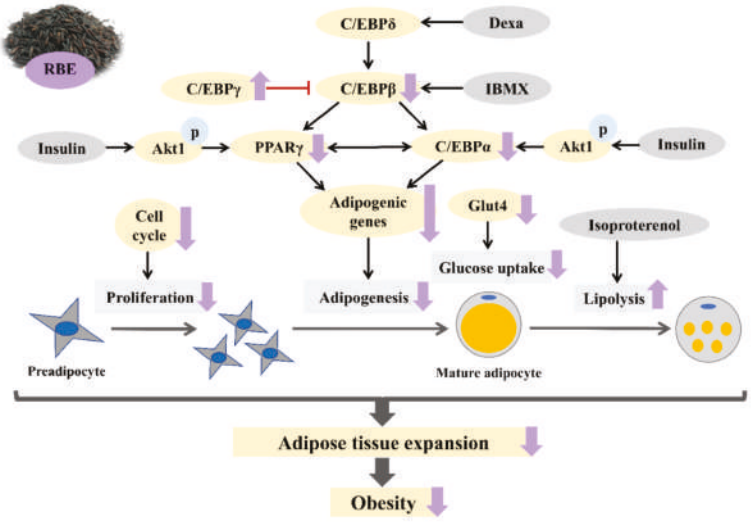

Figure 9. Schematic summary of the inhibitory effect of Riceberry rice extract (RBE) on cell proliferation and differentiation in 3T3-L1 adipocytes. RBE suppressed preadipocyte proliferation through inducing cell cycle arrest. RBE downregulated adipogenic transcription factors and their target genes, leading to inhibit adipogenesis and reduce glucose uptake. In addition, RBE enhanced isoproterenol-induced lipolysis in mature adipocytes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/8/2480/s1, Figure S1: Extracted ion chromatogram of phytochemical compounds detected in Riceberry rice extract (RBE) obtained by UHPLC–ESI-Q-TOF-MS/MS in negative and positive ion mode, Table S1: List of primers for RT-qPCR, Table S2: MS and MS/MS data of phytochemical compounds detected in Riceberry rice extract (RBE) obtained by UHPLC–ESI-Q-TOF-MS/MS in negative mode, Table S3: MS and MS/MS data of phytochemical compounds detected in Riceberry rice extract (RBE) obtained by UHPLC–ESI-Q-TOF-MS/MS in positive mode.

Author Contributions: P.K. was responsible for the study conception and design, funding acquisition, investigation, data analysis and interpretation, and preparation the original draft of manuscript. T.T. assisted with the methodology, experimental validation, and data analysis. M.M. performed the LC-MS/MS and data analysis. T.S., H.C. and S.A. contributed to the concept of the study, experimental design, findings interpretation, review and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ACC	acetyl CoA carboxylase
AdipoQ	adiponectin
AdipoQ-R1,R2	adiponectin receptor-1,2
aP2/Fabp4	fatty acid binding protein 4
ATGL	adipose triglyceride lipase
β-actin	Beta-actin
C3G	cyanidin-3-*O*-glucoside
$[Ca^{2+}]_i$	intracellular calcium concentration
CE	catechin equivalent
C/EBPα,β,γ	CCAAT-enhancer binding protein-alpha, beta, gamma
FasN	fatty acid synthase
Fura-2AM	Fura-2 acetoxymethyl ester
GAE	gallic acid equivalent
Glut4	glucose transporter 4
HSL	hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
InsR	insulin receptor
LPL	lipoprotein lipase
2-NBDG	2-(*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-Deoxyglucose
P3G	peonidin-3-*O*-glucoside
PPARγ	peroxisome proliferator-activated receptor-gamma
RBE	Riceberry rice extract
UHPLC-ESI-Q-TOF-MS/MS	ultra-high performance liquid chromatography coupled with electrospray ionization-quadrupole-time of flight-mass spectrometry

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Polyphenolic Maqui Extract as a Potential Nutraceutical to Treat TNBS-Induced Crohn's Disease by the Regulation of Antioxidant and Anti-Inflammatory Pathways

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Abstract: Nutraceuticals include a wide variety of bioactive compounds, such as polyphenols, which have been highlighted for their remarkable health benefits. Specially, maqui berries have shown great antioxidant activity and anti-inflammatory effects on some inflammatory diseases. The objectives of the present study were to explore the therapeutic effects of maqui berries on acute-phase inflammation in Crohn's disease. Balb/c mice were exposed to 2,4,6-trinitrobenzene sulfonic acid (TNBS) via intracolonic administration. Polyphenolic maqui extract (Ach) was administered orally daily for 4 days after TNBS induction (Curative Group), and for 7 days prior to the TNBS induction until sacrifice (Preventive Group). Our results showed that both preventive and curative Ach administration inhibited body weight loss and colon shortening, and attenuated the macroscopic and microscopic damage signs, as well as significantly reducing transmural inflammation and boosting the recovery of the mucosal architecture and its muco-secretory function. Additionally, Ach promotes macrophage polarization to the M2 phenotype and was capable of down-regulating significantly the expression of inflammatory proteins COX-2 and iNOS, and at the same time it regulates the antioxidant Nrf-2/HO-1 pathway. In conclusion, this is the first study in which it is demonstrated that the properties of Ach as could be used as a preventive and curative treatment in Crohn's disease.

Keywords: maqui; polyphenols; Crohn's disease; inflammatory bowel disease; acute inflammation; macrophages; antioxidants; inflammatory proteins

1. Introduction

Inflammatory bowel diseases (IBDs) are chronic idiopathic disorders of the gastrointestinal tract that mainly include two types: ulcerative colitis (UC) and Crohn's disease (CD) [1]. Their prevalence

has rapidly increased in the last decade, affecting five million patients worldwide, with the highest incidence in Northern Europe and Northern America [2]. Although the etiology is still unclear, it might be caused by various factors that could play a key role in the onset and progression of IBD, such as an exaggerated immune response to environmental factors, including the composition of the luminal microbiota in genetically susceptible individuals [3]. Particularly, CD is a complex disorder due to its variable locations, heterogeneous pattern of disease severity and behaviour, among others. A variety of proinflammatory mediators, including cytokines, oxidative stress (OS), disruption of the intestinal epithelial barrier and transmural inflammation can be seen in this illness. It can affect any portion of the digestive tract, although it commonly affects the ileum and the colon, and is marked by periods of exacerbation and remission, resulting in abdominal pain, diarrhea, weight loss and a generally poor quality of life [4]. The immune mechanism underlying CD pathogenesis is an aberrant adaptive immunity mediated by CD4+ helper T (Th) cells, specifically by Th17 and Th1, with an over-expression of IFN-γ, TNF-α, IL-2 and IL-12 production, together with suppression of the activity of T regulatory cells (Treg) [5,6]. Macrophages are an essential part of the innate immune system and provide important protection against harmful local antigens, such as those that cause intestinal inflammation. In tissues, macrophages are activated and produce multiple cytokines in response to various signals, and change to classical M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotypes. Upon stimulation, M1 phenotypes produce high levels of cytokines, such as TNF-α, IL-1β, IL-12, IL-18 and IL-23, chemokines (CXCL9, CXCL10), reactive oxygen species (ROS) and reactive nitrogen species (RNS), and are involved in driving Th1- and Th17- mediated immune responses. M2 macrophages, also referred to as 'alternatively activated', are marked by the expression of arginase-1 (ARG-1), IL-10 and IL-13, and have an immune-regulatory function [7,8]. The balance between inflammatory M1 and anti-inflammatory M2 cells could determinate the disease's progress, and therefore the factors implicated in the disruption of the balance toward an increase of the M2 macrophages cells could offer unique approaches for the future management of IBD [9].

The onset and progress of inflammation is directly related to two enzymes, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), associated with an increment of inflammatory lesions in the intestinal tissue [10]. In patients with CD, the higher immunoexpression of COX-2 in epithelial and inflammatory cells of the lamina propria has been observed [11], whereas in the inflamed gut of active ileocecal or colonic CD, iNOS is massively accumulated in subepithelial areas [12].

Interestingly, hemoxygenase-1 (HO-1) is one of the most important mechanisms of antioxidant defense, and its transcription depends on the nuclear erythroid 2-related factor 2 (Nrf-2), which is sequestered in the cytoplasm by the actin-binding protein Keap1, and it is responsible for the regulation of cellular redox balance. The presence of OS leads to the phosphorylation of serine/threonine residues in Nrf-2, to dissociate from Keap1 and translocation of Nrf-2 to the nucleus, and promotes binding to a specific DNA sequence known as the Antioxidant Response Element (ARE), resulting in an up-regulation of HO-1 [13]. Moreover, an over-expression of Nrf-2 has been observed in acute experimental models of colitis [14] and biopsies of inflamed tissue from patients with gastritis and IBD [15], leading to better inflammation regulation.

Furthermore, experimental colitis induced by hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS) is characterized by a predominant Th1/Th17-mediated immune response with elevated production of IL-12, IL-17, IL-18, IL-23, IL-27 and IFN-γ, and mucosal inflammation, which closely resembles important immunological and histopathological aspects of human CD [16,17]. In particular, the use of animal models is necessary to develop new therapies, since the conventional treatments for IBD (e.g., corticosteroids and immunomodulators) and biological therapies have many side effects and relative responses [18]. In this sense, nutraceuticals, including bioactive compounds such as polyphenols from berries with anti-inflammatory and antioxidant activities, could be potential agents for the treatment of IBD in humans [19]. Amongst them, *Aristotelia chilensis* (Mol) Stuntz (*Elaeocarpaceae)*, commonly known as maqui, is an edible black-colored fruit and endemic Chilean berry which has an exceptionally high content of phenolic compounds with high antioxidant capacity [20] and, less

studied, anti-inflammatory effects [21]. In a previous study, using a cellular model of OS consisting of hydrogen peroxide-treated (H_2O_2) HT-29 colon epithelial cells, we showed that our polyphenolic maqui lyophilized extract (Ach from now on) was rich in polyphenols and had a significant antioxidant effect, as evaluated through the intracellular ROS concentration using a DCFH-DA assay, protecting the intestinal epithelial cells against oxidation (unpublished).

The goal of the present work was to assess the effects of Ach on clinical, histological and inflammatory parameters using an animal model of acute phase of CD. We have analysed the preventive and curative effects of Ach during early colonic inflammation in mice caused by TNBS. Specifically, we have investigated the effect of Ach through macroscopy and histological study, as well as examining the implications for macrophage frequency and phenotype (M1 and M2). Furthermore, we determined, by Western blotting, the anti-inflammatory and antioxidant mechanisms through the expression of iNOS and COX-2, and the HO-1/Nrf-2 signaling pathway, respectively, in the colon mucosa of mice.

2. Materials and Methods

2.1. Extract

In order to obtain an extract including total polyphenols, the extraction was performed using the acid MeOH, according to the method published by Genskowsky et al. [22], with slight modifications. The initial sample consisted of 50 g lyophilized powdered wild maqui fruit (seed and pulp) from a packaged and commercialized product (Isla Natura de Chile®, Chiloé, Chile). In total, 250 mL of MeOH/H^+ (0.1% HCl) at pH 1 were added to the sample, and then homogenized with an ultrasound device (Hielscher Ultrasound Technology UP400S, Teltow, Germany). The extract was centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatant was collected. This procedure was repeated 5 times. The supernatants were mixed in a round-bottomed flask and evaporated until dryness using a rotary evaporator (Büchi B-490, Hampton, VA, USA). The dried extract was dissolved in distilled water and centrifuged at 4000 rpm for 4 min. Finally, it was passed through 2 filtering processes (100–150 MM and 40–100 MM) and then lyophilized in a Telstar Cryodos Freeze Dryer (Tokyo, Japan). The final extract was stored at −20 °C. All experiments were carried out under darkness and controlled temperature.

2.2. Animals

Male Balb/c mice aged 12–14 weeks old, weighing 19–35 g, were kept in pathogen-free cages and controlled laboratory conditions (temperature 20 ± 2 °C, humidity 40–50%, lighting regimen of 12 light/12 dark hours). Animals were fed a normal laboratory diet (Teklad diet, Envigo, Cambridgeshire, UK) and fresh tap water, ad libitum. The study was approved by the Ethical Committee of the Faculty of Medicine, University of Seville (name of the institution: 'Consejería de agricultura, pesca y desarrollo rural, Junta de Andalucía'; approval date: 19 January 2017; approval number: 03/03/2017/032). Animal handling was conducted in accordance with the Guide for the Care and Use of Laboratory Animals [23].

2.3. Induction of Colitis and Treatment

Animals were randomly assigned to 4 groups (n = 6). After fasting for 12 h, animals were anesthetized with ventilatory anesthesia and CD was induced according to the method reported by Liu T.J. et al. [24] with modifications. A single dose of TNBS at a concentration of 100 mg/kg was dissolved in ethanol (EtOH 50%) and installed intra-rectally, using at a total volume of 70 µL. The EtOH of this solution acts not only as the vehicle but also breaks the mucosal barrier and TNBS haptenizes colonic proteins, turning them immunogenic to the host's immune system [17]. Polyphenolic maqui extract (Ach) was administered daily, with a single dose of oral gavage of Ach at 50 mg/kg/day, for 4 days after TNBS administration (Curative Group) and for 1 week prior to the induction of the disease, until sacrifice (Preventive Group) (Figure 1).

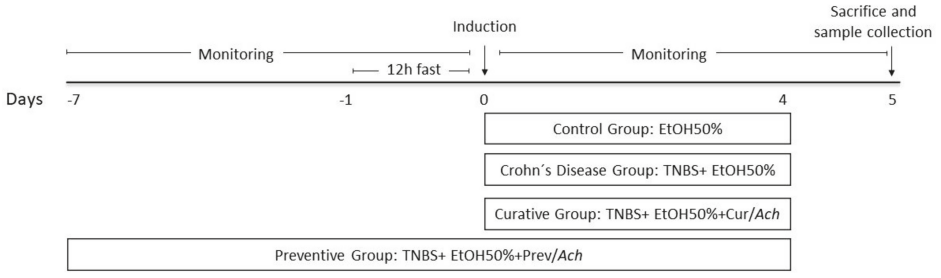

Figure 1. Schematic representation of experimental Crohn's disease (CD) protocols and treatments with polyphenolic maqui extract (Ach). 100 mg/kg of TNBS plus EtOH 50% was administrated to induce CD (CD Group). Mice were treated with 50 mg/kg/day of Ach 4 days after CD induction (Curative Group), and 7 days before and 4 days after induction (Preventive Group). The whole colon was collected at 5 days after colitis induction for determination of the microscopic score, macroscopic damage, macrophage polarization, and inflammatory and antioxidant pathway activation. Monitoring of weight and clinical parameters was performed during the experiment.

2.4. Macroscopic Evaluation of Colonic Damage

Mice were sacrificed 5 days after the onset of the experiment under intraperitoneal anesthesia. Once the death of the animals was confirmed by the absence of a response to a toe pinch and by touching the cornea, the abdominal cavity was opened, the entire large intestine was removed and lightly cleaned using physiological saline to remove fecal residues, and then was measured. Images of the colonic morphology were captured using a Canon EOS 350 zoom camera (Canon Inc., Tokyo, Japan). Thereafter, the intestine was opened longitudinally, and the macroscopic damage score was assessed by an independent observer, who was unaware of the groups' code. Damage was scored according to a modified version of the Appleyard and Wallace score [25]. Alterations in the intestinal mucosa were scored on a 0 to 11 scale, and the following items were considered: absence of damage, focal or little hyperemia without ulceration, bowel wall thickening, ulceration and local inflammation, two or more ulcerated and inflamed areas extending >1 cm along the length of the colon, damage extending >2 cm in length, one point for each cm from 2 cm on the damaged area, mucus and diarrhea (absent or present), intestinal adhesions and congestion (0–2 score). The other longitudinal half of the colon was collected and frozen in liquid nitrogen for later analysis.

2.5. Histopathological Study

For the histopathological study, half of the entire length of the large intestine was rolled up from the distal to the proximal end in order to evaluate the whole organ and its characteristics in only one slide. The longitudinal fractions of the colon from different groups were harvested and fixed overnight with 4% buffered paraformaldehyde and embedded in paraffin. Thereafter, sections of tissue were cut at 4 μm on a rotary microtome (Microm HM 310, Thermo Scientific, MA, USA), mounted on glass slides and dried for 2 h at 60 °C before staining with different methods. All histology slides were blindly analysed by two pathologists using an Olympus microscope (Vanox AHBT3, Tokyo, Japan).

2.5.1. Hematoxylin & Eosin Staining

The tissue sections were deparaffinized, hydrated and stained with hematoxylin–eosin, according to standard protocols. All histology slides were examined for histological evaluation of colonic inflammation and characterization of histopathological changes. Analysis was performed to establish a microscopic score system based on one which was previously described, but with some modifications [25,26]. Criteria include inflammatory infiltrate, goblet cell loss, crypt density, submucosal infiltration (all categorized from 0–3, corresponding to absent, mild, medium and severe) and ulcerations, crypt abscesses and necrosis (scored 0 or 1, corresponding to absent or present).

2.5.2. Periodic Acid Schiff Staining

A periodic Acid Schiff (PAS) stain was used to evaluate mucosal mucin production and the presence of the goblet cells. Briefly, the tissue sections were treated with periodic acid at 0.5% for 5 min and washed with distilled water. Finally, they were treated with the Schiff's reactive for 20 min, and again washed and stained with Harris hematoxylin. The goblet cells were assessed in the whole colon tissue at a resolution of 1× and 10×.

2.6. Macrophage Immuno Histochemistry

Immunostaining was performed on a Ventana Bench Mark GX stainer (Ventana Medical Systems, Roche Group, Tucson, AZ, USA) with pre-diluted Ventana anti-CD-68, as a marker of M1 macrophages, and anti-CD163, as a marker of M2 macrophages; both rabbit monoclonal primary antibody, and the Ventana Optiview DAB IHC detection kit—according to the manufacturer's instructions—were used (Ventana Medical Systems). Nuclei were counterstained with hematoxylin. Positive cells from samples of each animal were quantified and averaged from ten fields at high magnification (40×).

2.7. Isolation of Cytoplasmic Proteins and Western Blot Assay

Frozen colonic tissues were randomly selected (4 per group), weighted and homogenized in ice-cold buffer (50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 5 mM ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetra acetic acid, 0.5 mM EDTA, 0.01 mg/mL leupeptin, 0.01 mg/mL pepstatin, 0.01 mg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM NaCl) in a proportion of 1:3. The homogenates were centrifuged at 12,000× *g*, 4 °C for 15 min, and the supernatants were collected and stored at −80 °C. The protein concentration of the colon homogenates was determined following the Bradford colorimetric method [27]. Aliquots of supernatants containing equal amounts of protein (30 μg) were separated on 10% acrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In the next step, the proteins were electrophoretically transferred onto a nitrocellulose membrane, stained with Ponceau red to check transfer problems and the equal loading of total proteins, and blocked with 5% BSA in Nonidet^{TM} at 0.5% and PBS. Then, they were incubated with specific primary antibodies: rabbit anti-inducible nitric oxide synthase (iNOS) (1:1000; Stressgen-Enzo Life Sciences, Farmingdale, NY, USA), rabbit anti-COX-2 (1:3000; Cayman Chemical, Ann Arbor, MI, USA), rabbit anti-Nrf2 (1:1000; Santa Cruz, Texas, USA) and rabbit anti-HO-1 (1:1000; Stressgen-Enzo Life Sciences, Farmingdale, NY, USA) overnight at 4 °C. After rinsing, the membranes were incubated with the horseradish peroxidase-linked (HRP) secondary antibody anti-rabbit (1:1000; Cayman Chemical, Ann Arbor, MI, USA) or anti-mouse (1:1000; Dako, Atlanta, GA, USA) containing blocking solution for 1–2 h at room temperature. To prove equal loading, the blots were analyzed for β-actin expression using an anti-β-actin antibody (1:1000; Sigma Aldrich, St. Louis, MO, USA). Immunodetection was performed using an enhanced chemiluminescence light-detecting kit (SuperSignal West Pico Chemiluminescent Substrate, Pierce, IL, USA). Then, the immunosignals were monitored using an Amersham imager 600 (Healthcare Life Sciences, Buckinghamshire, UK), and densitometric data were studied following normalization to the housekeeping loading control. The signals were analyzed and quantified using Image Processing and Analysis in Java (Image J, Softonic, National Institute of Mental Health, Bethesda, MD, USA), and expressed as percentages in respect to the Control Group.

2.8. Statistical Analysis

All values are expressed as arithmetic means ± standard error of the mean (SEM). Heterogeneity was tested by using Levene's test and the Shapiro–Wilk test for normality. When the variables were normally distributed, Student's t-test was used along with the Mann–Whitney test for non-normally distributed data for the comparison of 2 means. For the comparison of 3 means, the parametric value groups were analyzed by one-way analysis of variance (ANOVA), or Kruskal–Wallis for the non-parametric values, followed by Bonferroni's post hoc. p-values of <0.05, <0.01 or <0.001 were

considered statistically significant. Statistical analysis and comparisons among means were carried out using STATA software (version 12, 2011, StataCorp, TX, USA).

3. Results

3.1. Therapeutic Effects on Macroscopic Damage of Ach on TNBS-Induced Crohn's Disease

Intracolonic administration of TNBS successfully induced a decreased stool consistency, rectal bleeding and significative weight loss in untreated mice. During the experiment, mice from the CD group (TNBS+EtOH 50%) showed a remarkable weight loss at 24 h after colitis induction which continued with a downward trend until sacrifice, compared to the Control Group (EtOH 50%). When 50 mg/kg of Ach was administered by an orogastric tube, the Curative and Preventive Groups showed a slight loss of weight the day after induction, which was recovered during the experiment period (Figure 2A). At the end of the experiment, significant differences were observed in the length of the colon. TNBS-induced CD mice exhibited a considerably shortened large intestine ($p < 0.001$), as an indicator of tissue inflammation, compared to the Control Group. On the contrary, the administration of Ach in the Curative and Preventive Groups significantly restored the colon length ($p < 0.05$ and $p < 0.01$, respectively) (Figure 2B–C). In line with the clinical findings, macroscopic examination of the colonic mucosa of the TNBS-induced CD mice evidenced hyperemia, congestion and ulceration, accompanied with mucus and liquid stool inside the colon. Additionally, a greater number of adhesions of the colon to the adjacent organs in the CD Group were observed. Colonic morphology and macroscopic characteristics were significatively restored in all groups after treatment with Ach ($p < 0.001$) (Figure 2D).

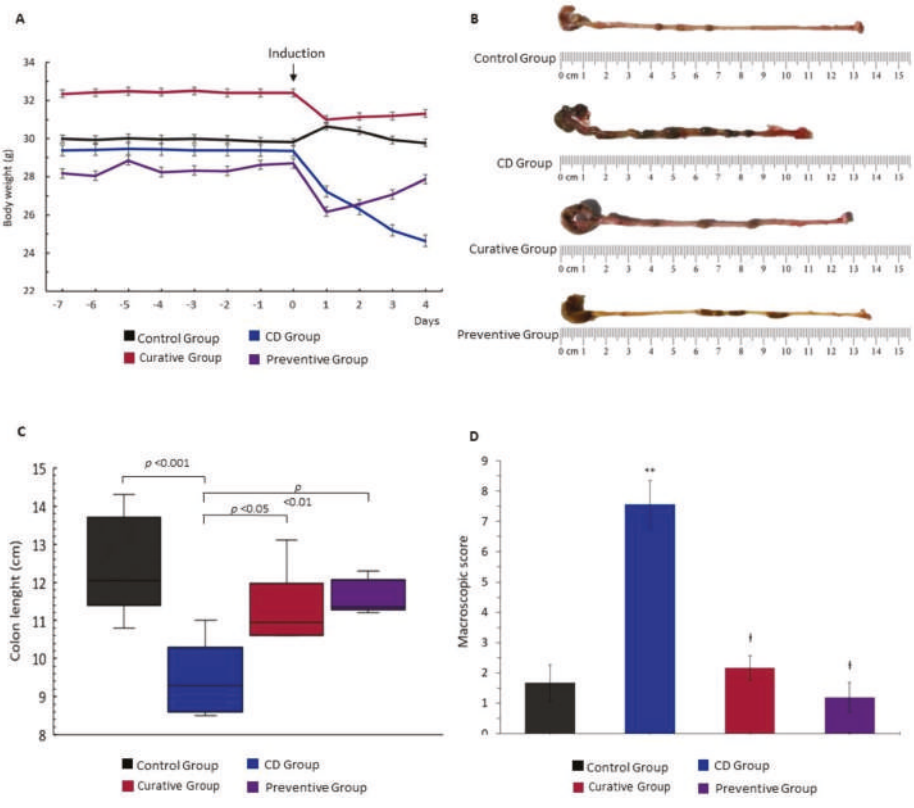

Figure 2. Polyphenolic maqui extract (Ach) prevents weight loss and ameliorates the macroscopic damage induced by TNBS in colon with Crohn's disease (CD). (**A**) Body weight changes during the experiment. (**B**) Representative view of large intestine from the Control Group, CD Group, Curative Group and Preventive Group. (**C**) Colon length of the different experimental groups. (**D**) Colonic macroscopic score. Values represent mean ± SEM; ** Statistical significance $p < 0.001$ compared to Control Group; ǂ $p < 0.001$ compared to CD Group.

3.2. Histological Effects of Ach on TNBS-Induced Crohn's Disease

The histopathological evaluation showed a transmural inflammation distributed in areas characterized by the loss of histological structure and a massive infiltration of inflammatory cells, specially neutrophils and lymphocytes associated with acute phase of inflammation, when compared with the Control Group. Mucosal and submucosal ulcerations, necrosis and edema in lamina propria were observed throughout the whole colon of the CD Group. After the administration of Ach (Curative and Preventive Group), the intestinal lesions revealed a reduction in inflammation, significant recovery of the pathological alterations, and the normal histological structure of the colon was re-established (Figure 3A–D). The histological score of the large intestine showed a significant increase ($p < 0.001$) in tissue damage in the untreated CD Group compared to the Control Group. Ach significantly reduced the histopathological score ($p < 0.001$) (Figure 3E), suggesting a protective and curative effect in the acute inflammation of CD. We also assessed PAS staining on colon tissue to determine the distribution of goblet cells and the presence of glycoproteins, including mucin content, within the colonic mucosa. TNBS-induced CD showed a negative staining for PAS (PAS-), indicating a significative reduction in the amount of goblet cells and mucin content. In contrast, the administration of Ach in the Preventive

and Curative Group exhibited a significant restoral to a normal pattern in colonic tissue form, similarly to Control Group, evidenced by the positive staining for PAS (PAS+) (Figure 4A–D).

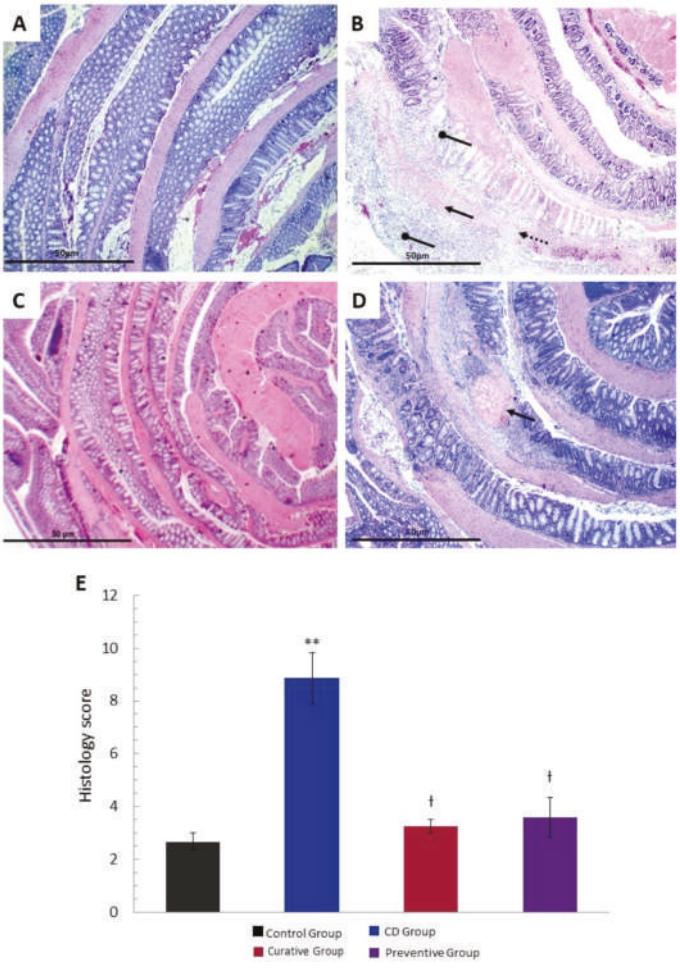

Figure 3. Preventive and curative effect of polyphenolic maqui extract (Ach) on microscopic evaluation and histopathological study of colonic tissue in a TNBS-induced acute Crohn's disease (CD) model. Representative hematoxylin/eosin (H&E) staining of the rolled entire large intestine (4× magnification). (**A**) Intact colonic tissue of the Control Group. (**B**) Severe loss of glandular architecture distributed in foci (rounded arrow), mixed inflammatory infiltrate (arrow; neutrophils and lymphocytes) and edema (dashed arrow) in the CD Group. (**C**) Recovered histological structure with mild inflammation in the lamina propria in the colon of the Ach-treated Curative Group. (**D**) Preserved architecture throughout the entire colonic tissue in the Ach-treated Preventive Group, except for a single focus of necrosis (arrow). (**E**) Histology score of large intestines from all groups. Values represent mean ± SEM** Statistical significance $p < 0.001$ compared to the Control Group; ǂ $p < 0.001$ compared to the CD Group.

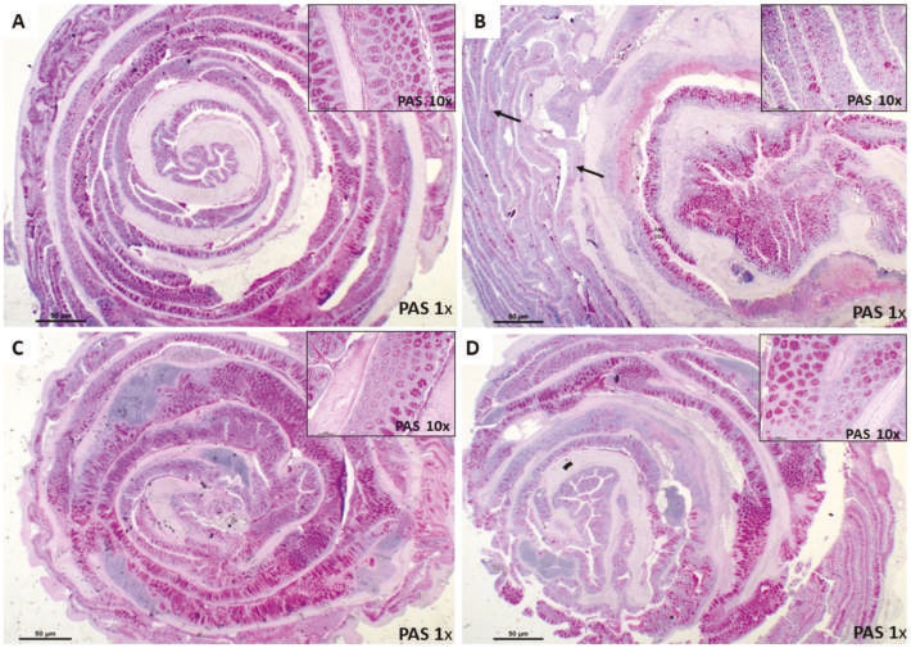

Figure 4. Preventive and curative effect of polyphenolic maqui extract (Ach) on the integrity of the colonic mucosa in a Crohn's disease (CD) murine model. Representative PAS staining for goblet cells in the rolled entire large intestines (1× and 10× magnification) of each group. (**A**) Control Group: strong PAS+ staining for glycoproteins, including mucins, showing functional colonic mucosa and crypts arranged in the usual position. (**B**) CD Group: poor staining with PAS in the middle and proximal area (arrows) (**C**,**D**) Curative Group and Preventive Group: PAS+ staining goblet cells distribution similar to the Control Group.

3.3. Impact of Ach on the Polarization to M1 and M2 Macrophages

Macrophage subtypes in the intestinal sections were visualized using immunohistochemistry. CD68+ (M1) and CD163+ (M2) macrophage cells were found within the layer of the lamina propria and submucosa in all groups. The amount of CD68+ cells was significantly higher ($p < 0.001$) in the CD Group compared with the Control Group, showing the activation of the innate immune response and the onset of the inflammatory process in CD. However, the Ach administration was not able to reduce the M1 macrophage expression, although we found a significative difference between the Curative and the Preventive Group ($p < 0.05$) (Figure 5A,B**).** It has been well established that M2 macrophages exert a modulatory role in TNBS-induced colitis [16]. Consequently, fewer M2 macrophages were expected to be found in the colonic tissue of TNBS-induced CD mice. On the other hand, we observed a significative increase in M2 cells in Ach treated mice (Curative and Preventive Group: $p < 0.01$ and $p < 0.001$, respectively) compared to the non-treated CD Group. Similar values were found in the Control Group, revealing a constant population of regulatory M2 macrophages in the colon (Figure 5C,D**).**

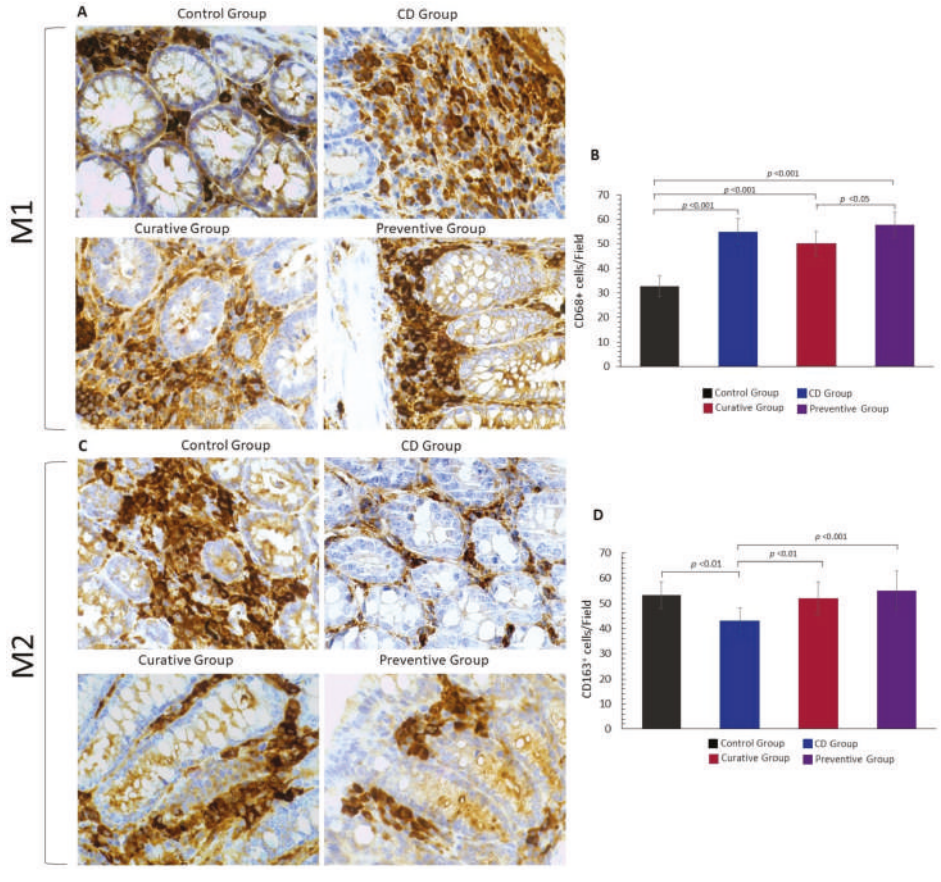

Figure 5. Polyphenolic maqui extract (Ach) restored M2 macrophage phenotypes in colonic tissue. (**A**) Immuno-histochemistry for $CD68^+$ macrophages (M1) in the large intestine from all groups. (**B**) Number of CD68+ cells counted from ten high-power fields in the colonic tissue of each group. (**C**) Immuno-histochemistry for $CD163^+$ macrophages (M2) in the large intestine from all groups. (**D**) Number of $CD163^+$ cells counted from ten high-power fields in the colonic tissue of each group. All immune-histochemical images are shown at 40× magnification. Values represent mean ± SEM.

3.4. Anti-Inflammatory Effect of Ach on the Protein Expression of iNOS and COX-2 Measured by Western Blot

To explore the possible mechanisms of the protective effect of Ach observed in the morphological and histopathological studies, we examined the expression levels of different inflammatory proteins in colonic mucosa. Western blot analysis showed that the TNBS-induced CD led to a significant increase in iNOS expression ($p < 0.05$), a pro-inflammatory enzyme related to the onset and perpetuation of inflammation [28]. However, the curative treatment with Ach after the induction with TNBS resulted in a significant down regulation of iNOS expression levels in comparison with the untreated group, while the Preventive Group showed a notable decrease in the iNOS expression (Figure 6A,B). As regards the COX-2 protein, widely known for acute inflammatory activity in epithelial and inflammatory cells [29], we observed an up-regulated expression trend in the CD Group. A significant decrease in COX-2 expression ($p < 0.05$) in the Curative and Preventive Groups was observed when compared with the CD Group (Figure 6A–C).

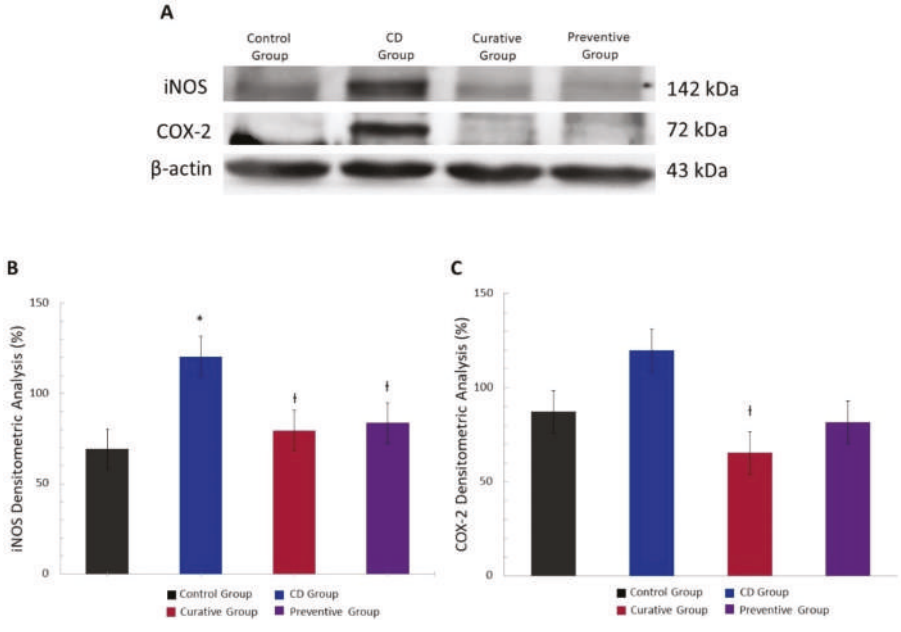

Figure 6. Anti-inflammatory effects of polyphenolic maqui extract (Ach) in the colon of mice from Crohn's disease (CD) model. (**A**) Representative Western blot images of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) pro-inflammatory proteins of each group. β-actin was used as an equal loading control for normalization. (**B**) Densitometric analysis of iNOS. (**C**) Densitometric analysis of COX-2. Values represent mean ± SEM; * Statistical significance $p < 0.05$ compared to Control Group; ǂ $p < 0.05$ compared to CD group.

3.5. Antioxidant Effect of Ach on Protein Expression of the Nrf-2/HO-1 Pathway, Measured by Western Blot

Based on the known antioxidant effect of Ach, Nrf-2/HO-1 pathway expression in the intestinal tissues was tested. As shown in Figure 7A,B, the level of Nrf-2 showed an over-regulation in the CD Group compared to the Control Group, without presenting significant differences. The Ach administration, in both the Preventive and Curative Groups, showed a slight upward trend in Nrf-2 protein expression. Likewise, HO-1 protein expression increased in TNBS-induced CD mice, with statistically significant values ($p < 0.05$). In line with Nrf-2 observations, HO-1 showed an increase when mice were given Ach, although this difference was not statistically significant (Figure 7A–C).

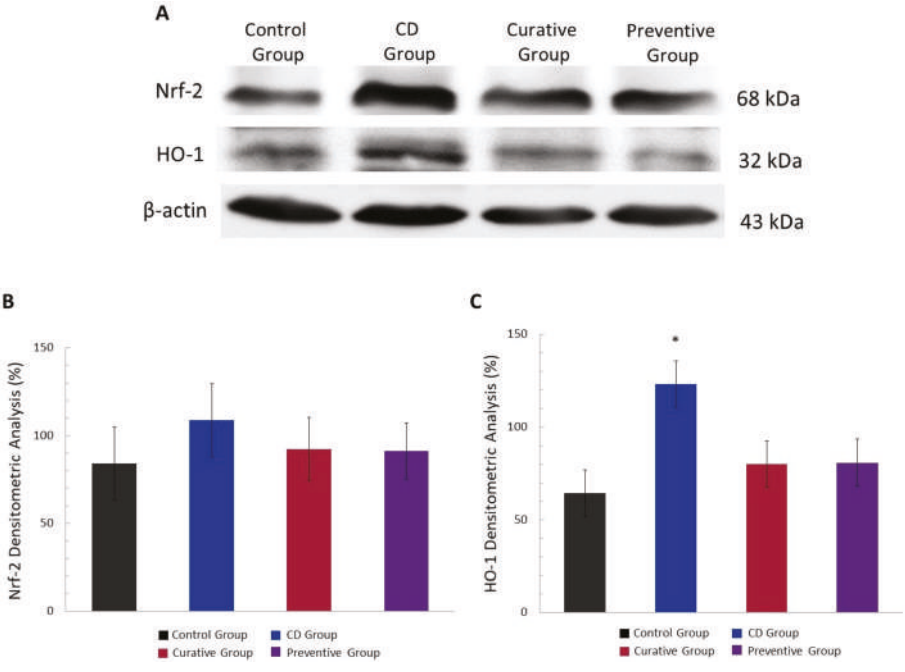

Figure 7. Antioxidant effect of polyphenolic maqui extract (Ach) in the colon of a TNBS-induced acute Crohn's disease (CD) model. (**A**) Representative Western blot images of the nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) and hemoxygenase-1 (HO-1) antioxidant proteins of each group. β-actin was used as an equal loading control for normalization. (**B**) Densitometric analysis of Nrf-2. (**C**) Densitometric analysis of HO-1. Values represent mean ± SEM; * Statistical significance $p < 0.05$ compared to Control Group.

4. Discussion

CD is characterized by chronic transmural and segmental intestinal inflammation that can affect any part of the gastrointestinal tract. The etiopathogenesis is complex and multifactorial, and is not totally clear yet [30]. Patients typically experience periods of flare-ups and symptomatic remission, which are difficult to predict and treat adequately [31]. Additionally, the standard medical therapy presents a relative efficacy, with a significant number of patients that fail or lose response during therapy and present potentially serious side-effects [18,32]. Due to the complexity of CD and the multitude of treatments, new therapeutic strategies, both effective and safe for IBD, have been reported. From this perspective, the use of nutraceuticals, including bioactive compounds such as polyphenols, has been gaining interest recently [33,34]. Maqui, a black-coloured edible berry, endemic to Chilean Patagonia, has been used in traditional medicine to treat various digestive disorders [35]. Indeed, this berry has been described as containing the highest antioxidant powers, with an extraordinary content of polyphenols, mainly delphinidin, and relevant anti-inflammatory properties [20,36]. However, the maqui berry's properties in the context of CD have not been researched yet. Regarding this, our extract has a total polyphenolic content of 39.02 mg/g (expressed as gallic acid), and we have found in previous in vitro experiments that polyphenolic maqui extract possesses powerful antioxidant effects in the H_2O_2-induced HT-29 epithelial cells (non published). Based on this background information, the aim of this study was to assess the preventive and curative effects of the administration of Ach on the development of the disease in the acute animal model of CD.

In our study, the intracolonic administration of 100 mg/kg of TNBS together with EtOH at 50% in a single dose was successful to induce CD in mice. The aim of the experimental parameters chosen,

such as dosage and exposure to TNBS, was to achieve acute inflammation and avoid a high mortality rate, which is frequently associated with higher concentrations than 150 mg/kg [37]. Although TNBS colitis does not reproduce perfectly the etiopathogenesis of CD, our results showed the development of multifocal and transmural inflammation, ulcerations, fibrosis and a loss of goblet cells. Previous articles have evaluated the effect of TNBS on the induction of colitis in experimental models, demonstrating its similarity to human CD [24,38,39]. Nevertheless, the presence of epithelioid granuloma, a specific histologic feature of CD, was not found. The etiology and significance of granuloma in CD are still unclear, but it is only seen in less than half of the cases of CD, and it could be associated with chronicity, according to other authors [5]. Additionally, the treatment dose with Ach was chosen according to previously published data in relation to polyphenols from natural sources and treatments in the intestinal affections of experimental animal [40,41]. However, most studies showed positive effects reducing inflammation and clinical parameters using higher concentrations than our study [42,43].

The results of this present study reveal, for the first time, the protective and curative effect of the administration of Ach in an experimental model of TNBS-induced CD. The administration of 50 mg/kg of Ach enhanced clinical parameters (diarrhea and blooding), ameliorated body weight loss and was able to improve macroscopic characteristics and tissue inflammation markers, such as the length of the large intestine, in TNBS- exposed mice. The equivalent amount of fresh maqui fruit for the dose administered to the mice was 100 mg/day. This value, translated to a standard human weight, corresponds to 240 g of fresh fruit per day.

Consistent with our findings, Scarano et al. [44] reported that some foods enriched with different polyphenols were able to reduce clinical damage and disease severity in an experimental colitis model, while other total polyphenolic extracts, derived from natural sources, have shown improvements in clinical parameters like weight and macroscopic damage, which were achieved at concentrations of 200 mg/kg of extract [42], which represented a higher quantity than that administered in our study. In the histopathological analysis of our study, the administration of Ach influenced the significant reduction of TNBS damage in the colon epithelial tissue of acute CD, and decreased the infiltration of inflammatory cells into the mucosa and adjacent layers, which have been suggested to contribute significantly to mucosal dysfunction and necrosis of intestinal wall tissue [45]. The protective effect of polyphenols on the architecture of the crypts and the production of mucus in goblet cells has previously been reported [46]. We demonstrated that the oral administration of Ach significatively recovered the mucosal architecture and its muco-secretory function, as evidenced by the PAS positive cells in the colonic mucosa.

A proinflammatory phenotype of M1 macrophages supporting Th1 cells contributes to the secretion of proinflammatory cytokines, as well as ROS and RNS. Alternatively, activated or M2 macrophages, which are anti-inflammatory and immunoregulatory, are polarized by Th2 cytokines and produce anti-inflammatory cytokines [47]. It has been previously reported that polyphenols exert an anti-inflammatory action by modulating macrophage phenotypes in inflammatory diseases [48,49], but not in IBD as yet. Additionally, the effect of other bioactive compounds was also reported to induce the polarization of M1 towards M2 macrophages, reducing systemic cytokines and attenuating colitis symptoms [8,50]. The local impact of Ach on macrophage differentiation was also studied in this work. We specifically demonstrated that macrophages responded to TNBS upregulating M1 and downregulating M2 macrophages. When a rich polyphenol source like Ach was administrated, the stronger effect was observed in M2 macrophages with high immunoexpression, attenuating or preventing the development of TNBS-induced CD. These results could indicate that M2 macrophages were more susceptible to modulation after Ach treatment, enhancing the anti-inflammatory effects of this treatment. A significative reduction of M1 could probably be achieved in a chronic model of CD. In support of this finding, it has been reported that the lamina propria monocytes and M1 macrophages contribute to the disruption of the epithelial barrier, leading to chronic intestinal inflammation in patients with IBD [12].

Moreover, other mechanisms described in IBD are mainly related to inhibiting the expression of inflammatory proteins, such as iNOS and COX-2 [51]. Nitric oxide (NO•), a free radical, is generated from the oxidation of L-arginine by its three enzymatic sources, which have been localized in the gastrointestinal tract. However, the large amounts of NO• generated from iNOS have been implicated in tissue damage and intestinal inflammation [52]. On the other hand, evidence from experimental models of intestinal inflammation demonstrate an up-regulation of iNOS expression [10,53]. In the present research, we observed a significative decrease in the expression of iNOS protein in the Preventive and Curative treatment groups with Ach. Our results are in agreement with previous in vitro studies that showed the capacity of maqui to modulate inflammatory response through inhibition of iNOS expression, with the consequent decrease of NO• levels [21,36]. The COX-2 enzyme catalyzes the formation of pro-inflammatory prostaglandins, thromboxane and levuloglandinas that are directly associated with inflammatory lesions in the colon of murine model CD [10,46]. The relation between COX-2 immuno-expression in inflammatory cells of patients with CD and the pathogenesis of CD has been reported [11]. In this sense, the induction of CD by TNBS induces acute inflammation symptoms that are accompanied by COX-2 colonic over-expression in relation with the Control Group. Our data evidenced a reduced COX-2 expression in mice treated with preventive and curative oral Ach. These findings, at least in part, suggest that Ach may be involved in the suppression of iNOS and COX-2, and could be considered for the treatment of IBD, specifically in CD. In fact, a recent study has identified that the ethyl acetate fraction of maqui berry water extract considerably reduces the expression of COX-2 and iNOS in DSS-induced UC [54].

During the inflammatory processes, the OS plays an important role in promoting its maintenance throughout time. In this condition, there are well-established increases of Nrf-2 expression and the consecutive regulation of the expression of numerous cytoprotective genes encoding antioxidant enzymes, such as ferritin, superoxide dismutase (SOD), peroxiredoxin-1 (PRDX1) and gluthathione S-tranferases (GSTs) [13,55], and also its downstream target, HO-1, to ameliorate the oxidative effects [56,57]. In our study, an Nrf-2 and HO-1 up-regulation was observed in the CD Group, indicating the primary events of acute inflammation, probably as a compensatory system to alleviate inflammation and tissue damage. On the contrary, Ávila-Román et al. [57] demonstrated reduced Nrf-2 and HO-1 expression levels in colon samples from a TNBS chronic colitis model. In addition, several dietary phytochemicals, such as green tea polyphenol components [58], anthocyanins [59] and carotenoids [56], have been implicated in reversing inflammatory processes by increasing the expression of Nrf-2 and HO-1. Our research has revealed that Ach administration generated an upward trend in this antioxidant pathway, which may prevent ROS production. It is also possible that these effects may be significant in the long term, specifically in a recurrent model, and other antioxidant enzymes regulated by Nrf-2 could be involved during the first phase of oxidative and inflammation injury. Moreover, a recent relevant report showed that maqui extract could exert an important effect on the microbiota imbalance in IBD [54]. Our findings propose to consider Ach as a potential nutraceutical for preventive or curative treatment in acute phase inflammation in CD, modulating the oxidative status and inflammatory response.

5. Conclusions

In summary, for the first time, it has been demonstrated that mice that have received Ach preventively, once the disease has started, are protected from TNBS-induced CD. The oral administration of Ach prevents weight loss and drastically reduces colonic tissue damage. Furthermore, we have demonstrated a reduction of transmural inflammation, and an increase in mucosal architecture recovery and its muco-secretory function. It is likely that Ach mediates this anti-inflammatory activity through multiple pathways. Our study has provided evidence that Ach promotes macrophage polarization to the M2 phenotype. In addition, we observed that Ach achieves protective effects against intestinal damage induced by TNBS through reduction of pro-inflammatory proteins levels such as COX-2 and iNOS, as well as regulation of antioxidant responses through the Nrf-2/HO-1 pathway. These results

indicate that Ach could be a novel approach to prevent and modulate the inflammatory response in IBD, especially in CD. Consequently, the clinical translation of these findings should be evaluated in the future.

Author Contributions: Conceptualization, M.D.-M., T.O. and J.-M.G.-M.; Formal analysis, T.O.; Funding acquisition, F.A.-A. and V.M.; Investigation, T.O., M.I., L.M.-G. and V.V.-R.; Methodology, T.O., M.I., J.-M.G.-M., A.A. and M.D.-M.; Project administration, M.D.-M.; Resources, F.A.-A., A.A., V.M. and M.D.-M.; Supervision, E.T. and M.D.-M.; Validation, L.M.-G.; Writing—original draft, T.O.; Writing—review and editing, F.A.-A. and M.D.-M. All authors have read and agree to the published version of the manuscript.

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Conflicts of Interest: The authors confirm that they have no conflict of interest.

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Vaccinium macrocarpon (Cranberry)-Based Dietary Supplements: Variation in Mass Uniformity, Proanthocyanidin Dosage and Anthocyanin Profile Demonstrates Quality Control Standard Needed

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Abstract: *Vaccinium macrocarpon* (syn. American Cranberry) is employed in dietary supplements (DS) with the aim to improve urinary tract well-being. This property is linked to the antiadhesion-activity of proanthocyanidins (PACs) against uropathogenic-bacteria. However, the current European legislation has been criticized for being weak and ineffective. Indeed, recent scientific works report mislabeled, contaminated, and adulterated supplements containing dangerous or unknown compounds, or sold at toxic doses. In this work, we analysed 24 DS that claim to contain cranberry, and to have a specific dosage of PACs. Our tests included the control of the good manufacturing practice according to the European Pharmacopoeia, and the verification of the claimed dosage of PACs. Moreover, in order to confirm the real presence of cranberry in DS, chemical fingerprinting via HPLC-UV/Vis-MS/MS was employed. Our results showed that 17 DS did not comply with the uniformity test of dosage forms, and only five contained cranberry. Finally, 16 DS claimed an incorrect amount of PACs. These data suggest that several cranberry-based DS are present in the European market with insufficient quality controls. Considering that often DS are self-prescribed by consumer relying on their claim, the data obtained in this work should encourage more controls and stricter rules.

Keywords: American cranberry; HPLC-UV/Vis; Orbitrap; Anthocyanins; Proanthocyanidins; chemical fingerprinting; dietary supplements; *Vaccinium macrocarpon*; BL-DMAC

1. Introduction

In recent years, an ever-increasing demand for supplements based on natural products has been recorded in Europe [1], and currently their sales are close to 7 billion Euros annually [2]. In particular, Italy ranks as the leading country in Europe for the consumption of plant-based dietary supplements (DS) [3]. These products are not simply used to support sport performances [4], but their non-prescribed use is very frequent also among children and adolescents [5], students [6], pregnant and postmenopausal women [7,8], and oncological patients or those affected by other chronic diseases [9,10].

However, the current regulation and monitoring systems of DS has been criticized for being weak and ineffective [11]. Indeed, the requirements for the quality control of food and drugs are not mandatory for DS [12]. On one hand, before the registration and introduction into the European market, medicines have to be subjected to scientific evaluation by the European Medicines Agency (EMA),

which involves clinical trials to probe their safety, quality, and efficacy [13]. On the other hand, the food safety is controlled by the European Commission, which established a legal framework to regulate the market of food, fortified food, and for so-called functional foods [14]. Moreover, according to the legislation, information about food derivation and origin must be unblemished and unquestionable for the consumer [15].

In this context, food supplements are considered as food, thus their registration, clinical trials, effectiveness, and toxicity testing are not required as they are for medicines or synthetic drugs [16]. However, food supplements are designed to look like synthetic drugs, since they have similar unit-doses and dosage [17]. Moreover, they are sold in pharmacies and, as with medicines, inadvertent overdosing may occur if the recommended posology and dosage are not strictly followed [17].

This borderline situation follows in the marketability of some DS that fail to declare all their active substances [18], the plant parts used for their formulation [19], or the professed amount of active substances [19,20] on their label. Additionally, in several cases a misidentification of the initial plant raw material, or the adulteration with other plants [21] is also found. Furthermore, in some cases, contaminations from environmental chemicals, including heavy metals, pesticide and herbicide residues [22], and mycotoxins and harmful micro-organisms [23,24] have also been reported. Since consumers perceive food supplements as natural, and consequently safe [25], the investigation of their quality and security is very important before their placing on the market.

In this study, we focused our attention on the quality control of 24 European bestselling *Vaccinium macrocarpon*-based DS. All these products share the claim to produce beneficial effects for the well-being of the urinary tract. Indeed, it has been demonstrated that a specific dosage of Cranberry's proanthocyanidins (PACs), quantified via BL-DMAC assay (Brunswick Laboratories 4-dimethylaminocinnamaldehyde), is sufficient to explicate a significant beneficial effect linked to its antiadhesion activity against uropathogenic P-fimbriated *Escherichia coli* bacteria [26].

In order to evaluate the proper formulation and dosage of the unit doses, mass uniformity, dosage uniformity, and PAC content were evaluated. Finally, in order to properly identify the used raw material, a chemical fingerprinting of all products was performed.

2. Materials and Methods

The 24 analysed DS comprise the bestselling products, according to the databases reporting the sell-in of medicine, pharmaceutical, and botanical products, including Cranberry-based DS.

2.1. European Pharmacopoeia Test for Mass Uniformity

The mass uniformity of the individual unit doses contained in each DS package was done following the European Pharmacopeia directives [27]. Briefly, at least twenty unit doses were taken, and each of them was individually weighted. Then, the average mass and its standard deviation was calculated. The unit doses complied with the test if not more than one individual mass was outside the 5% (tablets) or 7.5% (capsules and sachets) of the average mass. Moreover, tablets and capsules failed to comply with the test if one individual mass was outside the limits of 90%–110% (tablets) or 85%–115% (capsules and sachets) of the average mass.

2.2. Extract Preparation and Evaluation of Total Proanthocyanidin Content (tPAC)

For tPAC evaluation, different extract types were prepared depending on the pharmaceutical form of each DS unit dose. In particular, tablets were pulverized and then extracted with 30 mL of PAC Extraction Buffer [75% (*v*/*v*) Acetone with 0.5% (*v*/*v*) Acetic Acid] [28]. If present, the coating was manually removed from tablets before the extraction. Capsules were simply opened, and their total content was sampled and extracted with 20 mL of PAC Extraction Buffer. Sachets were opened and their whole content was extracted with 40 mL of PAC Extraction Buffer. Finally, liquids were directly diluted in 30 mL of PAC Extraction Buffer. All the extracts were vortexed for 5 min, and then placed into an ultrasound bath at room temperature for 30 min. The solution was further mixed on a

swing plate for 1 h. Samples were then centrifuged at 5000× *g* for 5 min, and the supernatant was collected and used for the quantification of tPAC via BL-DMAC assay [28]. In order to evaluate the completeness of the extraction process, the residues were also assayed. Extractions and quantifications were performed in triplicate.

2.3. Extracts Preparation and Evaluation of Total Anthocyanins Content (tTAC)

For tTAC evaluation, different type of extracts were prepared depending on the pharmaceutical form of each DS unit dose. In particular, tablets were pulverized and extracted with 20 mL of TAC Extraction Buffer [50% (*v*/*v*) Methanol with 1% (*v*/*v*) of Formic Acid] [29]. If present, the coating was manually removed from the tablets before extraction. Capsules were simply opened, and their total content was sampled and then extracted with 10 mL of TAC Extraction Buffer. Sachets were opened and the whole content was extracted with 30 mL of TAC Extraction Buffer. Finally, liquids were directly diluted in 10 mL TAC Extraction Buffer.

All the extracts were vortexed for 5 min, and then were placed into an ultrasound bath at room temperature for 30 min. The solution was further mixed on a swing plate for 1h. Samples were centrifuged at 5000× *g* for 10 min, and the supernatant was then collected and used for the quantification of tTAC via pH differential method [29]. Briefly, twenty µL of each extract was added separately to 980 µL of 0.025 M potassium chloride (pH 1.0), or to 980 µL of 0.4 M sodium acetate (pH 4.5). In order to prepare 100 mL of 0.025 M potassium chloride (pH 1.0) buffer, 0.19 g of KCl were weighted, and the solution was adjusted to the final desired pH by using HCl. To prepare the same amount of 0.4 M sodium acetate (pH 4.5) buffer, 5.44 g of CH_3COONa were weighted, and the solution was adjusted to the final desired pH by using CH_3COOH or NaOH. The absorbance was measured at 535 nm and 720 nm for both solutions, using 50% (*v*/*v*) Ethanol with 1% (*v*/*v*) formic acid as a blank. The assay was performed in triplicate. The tTAC was calculated using the following equation:

$$tTAC = \frac{\left(\Delta Abs_{pH1} - \Delta Abs_{pH4}\right) \times MW \times DF \times 1000)}{\varepsilon \times l}$$

where: Δ_{Abs} is the difference in the Abs recorded at 515 nm and 700 nm, at both *pH* 1.00 and *pH* 4.5; *MW* and ε are respectively the molecular weight (449.2 g mol^{-1}), and the molar extinction coefficient (26.900 mL mM^{-1} cm^{-1}) of cyanidine-3-glucoside, which was used as the reference compound; *DF* is the sample dilution factor; l is the path length (1 cm). The total anthocyanins were expressed as mg of cyanidin-3-glucoside equivalents per dosage unit. In order to evaluate the completeness of the extraction process, the residues were also assayed. Limit of detection (LOD) and Limit of Quantification (LOQ) were calculated using calibration curves of pure cyanidine chloride standard (Extrasynthase®, France), ranging between 1–20 µg/mL. LOD and LOQ were calculated as previously reported [30,31].

2.4. Chemical Fingerprinting of V. macrocarpon by HPLC-UV-VIS Orbitrap LC-MS detection

In order to assess the raw material used for the formulation of *V. macrocarpon*-based DS, chemical fingerprinting was performed by HPLC-UV/Vis, as previously described by Brown and Shipley [32]. Moreover, in order to identify a possible sophistication, all the DS showing an anthocyanin profile not attributable to *V. macrocarpon* were additionally analysed by UHPLC system, coupled to a quadrupole Orbitrap-mass spectrometer. All the obtained chromatographic profiles were compared to those of authentic *V. macrocarpon* fruits.

2.4.1. HPLC-UV/Vis. HPLC

The system consisted of an Agilent Technologies 1100, coupled to a UV-VIS detector, and the separation was carried out at constant flow rate (1.3 mL min^{-1}) using 10% (*v*/*v*) formic acid (Solvent A) and 50% (*v*/*v*) methanol, acidified with 10% (*v*/*v*) formic acid (Solvent B). The elution method involved a multistep linear solvent gradient: 0–5 min, 10% B; 5–15 min linear increase to 45% B; 15–20 min

increase to 60% B, 20–25 linear decrease to 10% B. The total analysis lasted 25 min, with an equilibration time of 5 min. Injected volume of the samples was 20 μL of solution. The column was a reverse phase C18 Luna column (5μm, 150 × 4.6 mm i.d., Phenomenex, USA) maintained at 50 °C by an Agilent 1100 HPLC G1316A Column Compartment. The UV-VIS spectra were registered at 520 nm. The identification of each compound was carried out by the comparison of both retention time and UV-VIS spectra of authentic reference compounds and by using literature data.

2.4.2. Orbitrap LC-MS detection

A UHPLC (Dionex UltiMate 3000 Rapid Separation LC) system by Thermo Fisher Scientific (San Josè, California) equipped with an autosampler, and controlled by Chromeleon 7.2 software by Thermo Fisher and by Dionex Softron GmbH (Germering, Germany) was used. The UHPLC system was coupled to a quadrupole Orbitrap mass spectrometer (Q Exactive; Thermo Scientific), equipped with an electrospray ion source. The conditions for the detection of anthocyanins were optimized by infusion of a solution of cyanidin-3-O-glucoside and cyanidin-3,5-O-diglucoside in positive-ion mode. The instrumental set-up was under the following conditions: sheath gas flow rate 35 (arbitrary units); auxiliary gas unit flow rate 15 (arbitrary units); spray voltage 3.5 kV; S lens RF 50; capillary temperature 300 °C. Separations were carried out on a UHPLC Luna® column C18(2) 100 Å (150 mm × 2 mm 3 μm, Phenomenex, USA). The injection volume was 1.0 μL. Mobile phase composition: 1% (*v*/*v*) formic acid in water (Solvent A), 1% (*v*/*v*) formic acid in methanol (Solvent B), at a flow rate of 150 μL min^{-1}. The gradient was: 0–5 min, 10% B; 5–10 min linear increase to 45% B; 10–15 min hold 45% B; 15–20 min, linear increase to 60% B; 20–25 min, linear increase to 90% B, 25–28 min, hold 90% B, 28–32 min, linear decrease to 10% B, 32–35 min, hold 10% B. The MS detection was conducted in Full MS / dd-MS^2 (TopN), with positive ion polarity, together with PRM experiment to confirm data obtained. The mass resolution of the Orbitrap analyzer was 70000 and the scan range was 400–700 *m*/*z*. Inclusion mass list is reported in Supplementary Table S1. Mass accuracy was checked daily by injecting a multi-compound standard solution, whereas every 2 days, the analyzer was calibrated with mass accuracy standards. Compound identification was carried out by comparison of both retention time and mass-fragmentation partner of each compound.

2.5. Statistical Analysis

Each extract was analysed in triplicate. All results are expressed as mean ± standard deviation (SD). All statistical analyses, tables, and graphs were done using graphpad Prism v5 (GraphPad Software, San Diego, CA USA), SYSTAT version 10.0 (SPSS, Chicago, IL, USA), and Microsoft Excel.

3. Results and Discussion

3.1. European Pharmacopoeia Test for Mass Uniformity

The main objective of the European Pharmacopeia is to provide the guidelines of pharmaceutical dosage forms through a series of quality control tests. In particular, in order to avoid possible side effects or lack of effect, the uniformity of dosage unit must be satisfied. The term "uniformity of dosage unit" is defined as "the degree of uniformity in the amount of the drug substance among the dosage units" (European Pharmacopeia, 2002). It can be demonstrated by both the evaluation of drug content uniformity or by the evaluation of mass uniformity among the dosage units. Due to sophisticated and expensive instrumentations required for the chemical analytical testes, the mass uniformity is the parameter that can be more easily examined. The weight variation test is based on the comparison of the individual weight of the pharmaceutical forms with an upper and lower percentage limit. In particular, DS packages complied with the test if not more than one individual dosage unit was outside of the percentage established with respect to the average mass (5% for tablets and 7.5% for capsules and sachets) (Criteria 1), but they failed to comply with the test if at least one individual mass

was outside of the double of these limits (10% for tablets and 15% for capsules and sachets) (Criteria 2). Mass uniformity results are reported in Table 1.

Table 1. Mass uniformity test of the 24 *Vaccinium macrocarpon*-based dietary supplements. The pharmaceutical forms (FF) of each package were evaluated following three different criteria. Criteria 1: FF complied with the test if not more than one individual mass was outside of the percentage established of the average mass (5% for tablets and 7,5% for capsules and sachets); Criteria 2: FF failed to comply with the test if one individual mass was outside of the double of these limits (10% for tablets and 15% for capsules and sachets); Criteria 3: FF failed to comply with the test if the ratio between the claimed mass and the average mass exceeded the 2%. The column "%CV" reports the percentage difference of the measured weight compared to that declared in each package.

#	Weight (g)		%CV	Criteria 1	Criteria 2	Criteria 3
	Claimed	Average of Each FF				
1	3.00	2.9776 ± 0.0326	99.25%	yes	yes	yes
2	4.50	4.5126 ± 0.0473	100.28%	yes	yes	yes
3	4.00	3.9816 ± 0.0209	99.54%	yes	yes	yes
4	4.15	4.2152 ± 0.0332	101.57%	yes	yes	yes
5	5.00	5.0545 ± 0.0318	101.09%	yes	yes	yes
6	3.30	3.3309 ± 0.1732	100.93%	yes	yes	yes
7	3.50	3.4611 ± 0.0926	98.88%	yes	yes	yes
8	2.00	1.8799 ± 0.2519	93.99%	no	no	yes
9	3.50	4.1581 ± 0.6992	118.80%	no	no	no
10	0.90	0.3455 ± 0.0201	38.38%	no	no	no
11	0.35	0.3971 ± 0.0091	115.10%	yes	yes	no
12	0.53	0.5753 ± 0.0833	107.93%	no	no	yes
13	0.42	0.4392 ± 0.0131	104.07%	yes	yes	yes
14	0.50	0.5011 ± 0.0113	100.22%	yes	yes	yes
15	20.00	23.192 ± 0.5796	115.96%	yes	yes	no
16	1.20	1.1901 ± 0.0144	99.17%	no	yes	yes
17	1.20	1.0260 ± 0.0142	85.50%	no	yes	no
18	0.75	0.7478 ± 0.0063	99.70%	yes	yes	yes
19	0.55	0.5362 ± 0.0058	97.49%	yes	yes	yes
20	1.03	1.0554 ± 0.0052	102.46%	yes	yes	yes
21	0.97	0.9722 ± 0.0113	100.43%	yes	yes	yes
22	0.68	0.6901 ± 0.0194	102.23%	no	yes	yes
23	0.88	0.8747 ± 0.0093	99.96%	yes	yes	yes
24	1.15	1.1972 ± 0.0111	104.37%	yes	yes	yes

Among the observed DS, seven of them (#8, #9, #10, #12, #16, #17, and #22) had more than one dosage unit with a mass outside the 5% and 7.5% limit of their average mass. In particular, #8, #9, #10, and #12 had also at least one unit dosage with a weight outside the 10% and 15% range of their average mass. Moreover, some of the DS that did not satisfy Criteria 1 and Criteria 2, had an individual dosage unit mass outside of the 10% of the weight declared in the package label. Therefore, these DS, together with #15 and #17, did not satisfy Criteria 3. In particular, #9, #11, #15, and #17 showed an average mass 15% higher than that declared in the label, while #10 showed an average mass that was about 60% lower.

In conclusion, according to European Pharmacopeia (Criteria 1 and Criteria 2), only 17 of the 24 DS (#1, #2, #3, #4, #5, #6, #7, #11, 13, #14, #15, #18, 19, #20, #21, #23, and #24) satisfied the guidelines of the uniformity of pharmaceutical dosage, but two of them (#11 and #15) claimed, in the label, a weight of individual dosage unit that was largely different from the one empirically evaluated.

3.2. Evaluation of tPAC in V. macrocarpon-Based Dietary Supplements

Even though, in the past, the beneficial properties of cranberry was linked to bacteriostatic effect of its acidity [33], recent scientific data showed that this protective effect may depend on the antiadhesion

activity of PACs against uropathogenic bacteria [34,35]. In addition, a randomized clinical trial showed that a daily dose of at least 36 mg PACs measured via BL-DMAC assay is necessary to explicate a significative beneficial effect [36]. Consequently, the efficacy of these preparations was strongly related to the amount of PACs in the unit doses determined through this spectrophotometric assay. For this reason, almost all *V. macrocarpon*-based DS on the market, including those examined in this work, claim not only a specific PAC dosage, but also PAC quantification via BL-DMAC. This assay is considered to be the most rapid and accurate spectrophotometric method to determine the total content of PACs, considering all grades of their polymerization [28], and excluding the interference of anthocyanins [37]. Although currently other methods exist for PACs quantification, including those based on liquid chromatographic systems [38], the manufacturer preference to quantify PACs via BL-DMAC assay should not be surprising. Indeed, liquid chromatographic techniques, coupled with mass-spectrometer (LC-MS), may be very expensive, even requiring trained personnel that would increase production costs. Moreover, due to the enormous variability in PAC polymerization-degree, not every LC-MS system is able to carry out an accurate quantification of the total PAC amount [39–41].

In this study, we firstly evaluated if the observed DS contained PACs. For this estimation, we decided to perform BL-DMAC assay. On one hand, the results obtained by these quantifications were employed in order to check if the DS that claimed to have a certain PAC amount quantified via BL-DMAC assay actually had the stated dosage. On the other hand, all the DS were analysed by this assay in order to understand if, following the indication of the leaflet, the suggested dosage of 36 mg per day measured via BL-DMAC assay [36], was insured. Considering DS labels, PACs content was claimed in 16 (#1, #3, #4, #5, #6, #7, #8, #9, #10, #14, #16, #17, #18, #19, #20, and #24) of the 24 observed DS. However, only eight of them (#4, #5, #6, #8, #9, #10, #19, and #24) also declared that BL-DMAC was the analytical method used for PACs quantification. However, after the BL-DMAC assay, we revealed that all the observed DS contained PACs, including those that did not report it in the label (Table 2).

Among the DS that declared PAC content and BL-DMAC as analytical methods for their quantification, our results showed that #4, #5, #19, and #24 contained PACs amount comprised between the 95–105% of the declared value. On the contrary, a content 85% lower than that declared was determined in #9, but a tPAC lower than 15% of that declared was found in #6, #8, and #10.

Concerning both the supplements that declared PAC content without specifying the analytical method used for their quantification, and those that did not report it, our analysis were limited to the simple estimation of PACs in the unit doses. Moreover, BL-DMAC assay was also performed to evaluate if the suggested posology insured the daily intaking of 36 mg of PAC [36]. Our results showed that #3, #4, #5, #9, #19, #20, #22, and #24 were the only supplements in which the daily intaking of PACs was guaranteed. For other DS, the posology suggested in the label allows the achievement of a daily administration ranged between 0.56 and 12.96 mg of PAC.

Table 2. Quantification of total proanthocyanidins content (tPACs) in the 24 *Vaccinium macrocarpon*-based dietary supplements by BL-DMAC assay. The column "method" reports the claimed analytical method used by the manufacturer for the quantification of tPACs. Results are expressed as mean of three different replicates ± SD, using external calibration curves of dimer PAC-A type. In the column "%CV", the percentage differences of tPACs between the value declared and that calculated via BL-DMAC assay are reported for each product.

#	mg PAC per FF			%CV
	Claimed	Method	Real	
1	36		5.54 ± 0.27	15.39%
2	n.d.		7.45 ± 0.45	
3	43.2		41.66 ± 1.13	96.44%
4	36	BL-DMAC	34.86 ± 1.73	96.83%
5	18	BL-DMAC	17.84 ± 1.34	99.11%
6	36	BL-DMAC	4.91 ± 0.22	13.64%
7	50		4.41 ± 0.01	8.82%
8	18	BL-DMAC	2.48 ± 0.05	13.78%
9	45	BL-DMAC	37.89 ± 1.59	84.20%
10	46.8	BL-DMAC	0.55 ± 0.05	1.18%
11	n.d.		1.51 ± 0.09	
12	n.d.		2.79 ± 0.14	
13	n.d.		3.68 ± 0.19	
14	30		6.01 ± 0.23	20.03%
15	n.d.		1.13 ± 0.06	
16	18		0.93 ± 0.03	5.17%
17	36		5.31 ± 0.44	14.75%
18	20		6.48 ± 0.21	32.40%
19	14.4	BL-DMAC	13.92 ± 0.28	96.67%
20	54	BL-DMAC	32.91 ± 0.91	60.94%
21	n.d.		0.38 ± 0.02	
22	n.d.		17.12 ± 0.63	
23	n.d.		0.28 ± 0.02	
24	36	BL-DMAC	37.17 ± 1.53	103.25%

3.3. Evaluation of TAC in the 24 V. macrocarpon-Based Dietary Supplements

When powder extracts are used for the preparation of plant-based DS, they cannot be used for taxonomic examination to identify their starting raw material. Specific chemical markers instead can be useful to discriminate their plant origin. Cranberry fruits are characterized by a high amount of anthocyanins [42]. Therefore, anthocyanins should always be present in powder extracts that claim to be obtained from *V. macrocarpon* fruits. In order to evaluate TAC, the pH differential method was used. This method makes it possible to selectively determine the real content of anthocyanins in samples, excluding at the same time the contribution of others chemical colorants or red natural pigments [43].

Although our analysis showed that all the observed DS contained PACs (Table 2), five of the 24 *V. macrocarpon*-based DS (#8, #11, #12, #21, and #23) did not contain anthocyanins (LOD: 3 μg mL^{-1}; LOQ: 10 μg mL^{-1}). For all the other supplements, we recorded a TAC value ranging between 0.389 ± 0.032 to 21.977 ± 1.713 mg of anthocyanin per unit doses. All TAC values are reported in Table 3.

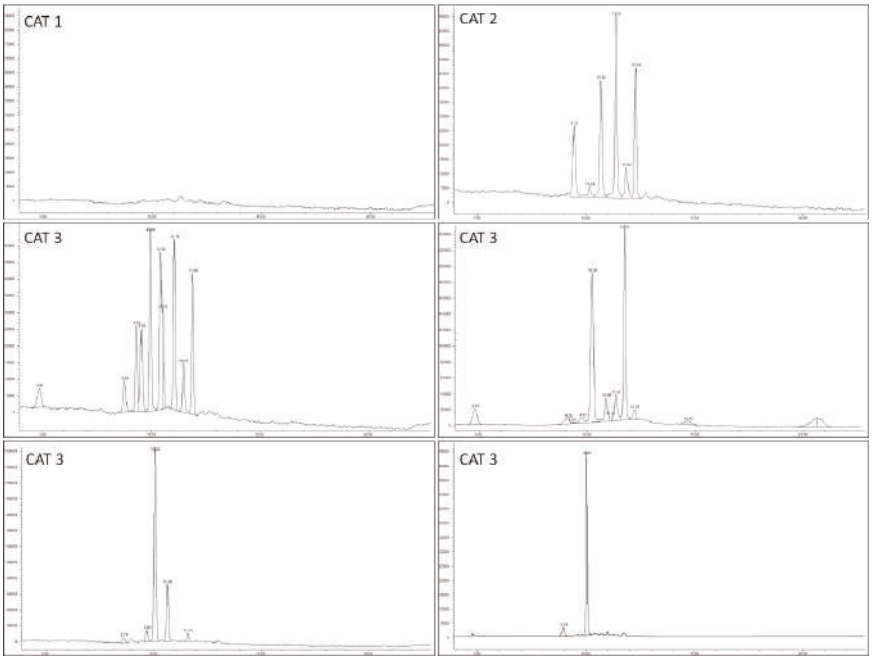

Figure 1. Representative chromatograms of the three main categories into which the 24 *Vaccinium macrocarpon*-based dietary supplements were grouped. CAT 1 groups the products that showed a flat chromatogram, indicating not only the absence of *Vaccinium macrocarpon*, but also of any other fruit containing anthocyanins; CAT 2 groups the products that showed a chromatogram typical of *Vaccinium macrocarpon* fruits; CAT 3 groups the products that contained anthocyanins not characteristic of *Vaccinium macrocarpon*.

Table 3. Quantification of total anthocyanin content (TAC) and chemical fingerprinting of the 24 *Vaccinium macrocarpon*-based dietary supplements. Results are the mean of three different replicates, and they are expressed as mg of cyanidine chloride equivalent ± SD. The different supplements are grouped in three different categories in the column "CAT", depending on the different chromatogram recorded at λ = 520 nm (see Figure 1). The non-typical anthocyanins found in each sample are reported in the column "UHPLC-Orbitrap". (D3Ru = delphinidin-rutinoside; D3Gl = delphinidin-glucoside; Pet3Gl = petunidin-glucoside; C3Ru = cyanidine-3-rutinoside).

#	TAC per FF	Chemical Fingerprinting						
		HPLC-UV/Vis			UHPLC-Orbitrap			
		CAT 1	CAT 2	CAT 3	D3Ru	D3Gl	Pet3Gl	C3Ru
1	3.601 ± 0.211		x					
2	21.997 ± 1.713			x	x	x		
3	5.462 ± 0.343			x	x	x		
4	13.317 ± 0.258			x				
5	8.925 ± 0.241		x					
6	2.322 ± 0.131			x	x	x		
7	0.389 ± 0.032			x	x	x		
8	n.d.	x						
9	19.501 ± 1.569		x					
10	4.859 ± 0.121			x	x	x	x	
11	n.d.	x						
12	n.d.	x						

Table 3. *Cont.*

#	TAC per FF	Chemical Fingerprinting						
		HPLC-UV/Vis			UHPLC-Orbitrap			
		CAT 1	CAT 2	CAT 3	D3Ru	D3Gl	Pet3Gl	C3Ru
13	6.325 ± 0.589			x				
14	0.444 ± 0.021			x	x	x		
15	4.359 ± 0.356			x	x	x		
16	2.134 ± 0.123			x				
17	1.134 ± 0.0458			x	x	x		
18	3.184 ± 0.192		x					
19	1.102 ± 0.0874			x	x	x		
20	6.439 ± 0.255			x	x	x	x	
21	n.d.	x						
22	2.907 ± 0.287			x	x			
23	n.d.	x						
24	3.361 ± 0.055			x	x	x		x

Considering anthocyanin bioactivity and pharmacological activity, the inclusion of TAC value in the claim of *V. macrocarpon*-based DS would be useful for a correct and complete labelling of these products, even though the current legislation does not foresee it.

3.4. Chemical Fingerprinting of the 24 V. Macrocarpon-Based Dietary Supplements by HPLC-UV/Vis and Orbitrap LC-MS analysis

Fingerprint techniques, including DNA molecular fingerprint [44] and chemical fingerprinting [45], are crucial for assessing quality of commercial products, and give information about the purity of the original raw material. DNA molecular fingerprinting cannot be applied to the major part of extracts made with organic solvent, due either to the insolubility of nucleic acids in extraction solvents or to DNA instability during manufacture procedures. In these cases, chemical fingerprinting is the best approach, allowing the research of specific chemical markers, which are known to be present in, or also absent from, a particular raw material. Currently, different chemical fingerprinting techniques can be used, with the aim to search for specific markers of the different raw materials. However, all these techniques employ chromatographic systems, but are not always coupled with mass spectrometers. For example, the presence of *V. macrocarpon* fruit in processed products may be checked through the analysis of the general phenolic compositions [46], or focusing on specific bioactive compounds, such as proanthocyanidins [47] or anthocyanins [48,49]. In this context, chemical fingerprinting based on proanthocyanidins or other polyphenols may be less accurate when DS are deliberately adulterated with plants with a similar phytochemical composition. On the other hand, the different anthocyanins have a specific ratio and chemical pattern of distribution among plant species. Consequently, their chemical profile can be useful for better identify the voluntary adulterations of DS [50]. However, the exclusive presence of anthocyanins cannot be considered a sure chemical marker without the identification and characterization of each anthocyanin compound. In particular, even though anthocyanins are one of the largest and most widely spread compounds in several genus of plant, each plant has a specific chemical pattern of distribution [50]. Moreover, although the fruit anthocyanin amount can change due to degradation along processing and storage, the qualitative anthocyanin profile is unique and characteristic [51]. For example, malvidin and pelagordin cannot be present in blackberry fruits [52]. In the same way, delphinidin and malvidin cannot be present in strawberry fruits [53]. On the other hand, *V. macrocarpon* fruits must contain five anthocyanins, for instance cyanidin-3-O-galactoside (C3Ga), cyanidin-3-O-glucoside (C3Gl), cyanidin-3-O-arabinoside (C3Ar), peonidin-3-O-galactoside (P3Ga), and peonidin-3-O-arabinoside (P3Ar) [32,54]. Despite being detected in very low amounts, delphinidin, malvidin, and their related-conjugated cannot be highly present in *V. macrocarpon* fruits [55]. Moreover, it should be considered that when food industry waste material was used for DS production

(i.e., the residue of the squeezing for the production of *V. macrocarpon* juices), the anthocyanin profile could be less visible, but still present.

Comparing the HPLC-UV/Vis chromatogram of the unknown samples to those of identified *V. macrocarpon* fruits, some important information about the raw material may be obtained [56]. The extracts of the observed 24 *V. macrocarpon*-based supplements were analysed by HPLC-UV/VIS in order to compare the anthocyanin profile with the typical *V. macrocarpon* chromatographic pattern. Based on DS chromatograms, the analysed supplements were gathered in three different groups. Figure 1 reports the representing chromatograms of the three different categories, meanwhile the classification of each DS across the three categories is reported in Table 3. The first category (CAT 1) groups the five DS (#8, #11, #12, #21, and #23) that showed a flat chromatogram (Figure 1, Panel A), thus confirming pH differential method data. The second category (CAT 2) gathers the four DS (#1, #5, #9, and #18) that displayed the same anthocyanin pattern of the *V. macrocarpon* fruits analysed in this experimentation (Figure 1, Panel B). Finally, all the others DS (#2, #3, #4, #6, #7, #10, #13, #14, #15, #16, #17, #19, #20, #22, and #24) displayed anthocyanin patterns different from those of *V. macrocarpon* fruits (CAT 3).

Due to the higher sensitivity of mass-spectrometer with respect to the UV/Vis detector, the UHPLC coupled with high resolution tandem mass spectrometer Orbitrap was employed to analyze all 24 DS. In particular, our analyses aimed to confirm either the absence of anthocyanins in the supplements listed in CAT 1, or the exclusive presence of those characteristic of *V. macrocarpon* fruits in the DS grouped in CAT 2. Finally, the supplements reported in CAT 3 were also analysed in order to confirm the absence of the anthocyanins typical of *V. macrocarpon* fruits.

The UHPLC-MS/MS data confirmed the HPLC-UV/Vis analysis, on the absence of any anthocyanin in the DS listed in CAT 1. On the other hand, #1, #5, #9, and #18 showed the same anthocyanin profile of *V. macrocarpon* fruits, and did not contain atypical anthocyanins. Finally, UHPLC analysis established the absence of *V. macrocarpon* typical anthocyanins in all the DS listed in CAT 3, except for #20. In this case, UHPLC-MS/MS analysis helped in detecting a low amount of *V. macrocarpon*'s anthocyanins, which was not possible to detect through HPLC-UV/Vis. However, #20, as well as all other supplements listed in CAT 3, showed unexpected chromatographic peaks. UHPLC analysis identified these peaks as delphinidin-rutinoside (#2, #3, #6, #7, 10, #14, #15, #17, #19, #20, #22, and #24), delphinidin-glucoside (#3, #4, #6, #7, #10, #13, #14, #15, #16, #17, #19, #20, and #24), petunidin-glucoside (#10 and #20), and cyanidine-3-O-rutinoside (#20). Even though Oszmiański and colleagues reported that delphinidin-derivates may be present only in small quantities with respect to cranberry-typical anthocyanins [55], the opposite situation is shown in all DS listed in CAT 3, as displayed by the output generated by HPLC-UV/Vis analysis.

4. Conclusions

In this paper, we showed how several *V. macrocarpon*-based DS currently on the European market displayed several problems during the formulation and manufacture processes. Indeed, 17 of the 24 DS were shown not to comply with the Pharmacopeia criteria of good preparation. Moreover, 16 of 24 DS claimed in the label to ensure an amount of PACs equal to, or greater than, that shown to be active for the healthcare of the urinary tract. However, only six of them had a tPAC equal to that declared in the label. Finally, the chemical fingerprinting displayed that only five DS contained *V. macrocarpon* extract, meanwhile, the misidentification of the raw material was clear in all the others. Among all the observed 24 *V. macrocarpon*-based DS, only one (**#5**) contemporary complied the criteria of good preparation, respected their uniformity of dosage, and contained *V. macrocarpon* (Table 4).

These data suggested that several *V. macrocarpon*-based DS are present in the European market with insufficient quality controls, and that they are frequently mislabeled. Considering that in most cases DS are self-prescribed, and that the consumer relies exclusively on the claim and label of these products, the lack of an appropriate control quality confuses the consumer in choosing a product that should contribute to his personal well-being.

Table 4. Summary table of the quality control tests carried out on 24 *Vaccinium macrocarpon*-based dietary supplements. The symbol "✓" marks the passing of the relative test for each product. On the contrary, the symbol "x" marks when the DS failed to comply with the test. Criteria 1: FF complied with the test if not more than one individual mass was outside of the percentage established of the average mass (5% for tablets and 7.5% for capsules and sachets); Criteria 2: FF failed to comply with the test if one individual mass was outside of the double of these limits (10% for tablets and 15% for capsules and sachets); Criteria 3: FF failed to comply with the test if the ratio between the claimed mass and the average mass exceeded the 2%. The column "36 mg/day" reports if, following the suggested posology, the administration of at least 36 mg of PACs was guaranteed, while the adjacent column reports if the dosage claimed in the label was correct. The other columns respectively report the detection of anthocyanins (TAC), and whether the anthocyanin pattern was different from those of the *V. macrocarpon* fruits analysed in this experimentation (FP).

	Uniformity of Mass			PACs Content		Cranberry	
#	Criteria 1	Criteria 2	Criteria 3	36 mg/day	Correct Dosage	TAC	FP
1	✓	✓	✓	x	x	✓	✓
2	✓	✓	✓	x	n.r.	✓	x
3	✓	✓	✓	✓	✓	✓	x
4	✓	✓	✓	✓	✓	✓	x
5	✓	✓	✓	✓	✓	✓	✓
6	✓	✓	✓	x	x	✓	x
7	✓	✓	✓	x	x	✓	x
8	x	x	✓	x	x	x	x
9	x	x	x	✓	x	✓	✓
10	x	x	x	x	x	✓	x
11	✓	✓	x	x	n.r.	x	x
12	x	x	✓	x	n.r.	x	x
13	✓	✓	✓	x	n.r.	✓	x
14	✓	✓	✓	x	x	✓	x
15	✓	✓	x	x	n.r.	✓	x
16	x	✓	✓	x	x	✓	x
17	x	✓	x	x	x	✓	x
18	✓	✓	✓	x	x	✓	✓
19	✓	✓	✓	✓	✓	✓	x
20	✓	✓	✓	x	x	✓	✓
21	✓	✓	✓	x	n.r.	x	x
22	x	✓	✓	✓	n.r.	✓	x
23	✓	✓	✓	x	n.r.	x	x
24	✓	✓	✓	✓	✓	✓	x

n.r. = PACs was not reported in the label of DS

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/12/4/992/s1, Table S1: Inclusion mass list of anthocyanins that are not characteristic of *Vaccinium macrocarpon* fruits and that were researched in the 24 cranberry-based dietary supplements. Figure S1–S24: HPLC-UV/Vis analysis of the 24 cranberry-based dietary supplements.

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Review

Polyphenols and Human Health: The Role of Bioavailability

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Abstract: Polyphenols are a group of phytochemicals with potential health-promoting effects. They are classified as flavonoid (flavonols, flavanols, flavones, flavanones, isoflavones, and anthocyanins) and non-flavonoid molecules (phenolic acids, hydroxycinnamic acids, lignans, stilbenes, and tannins). Although an increasing number of trials have shown a correlation among polyphenol consumption and a reduction in risk factors for chronic diseases, discrepancies in explaining their positive effects have been found in terms of the bioavailability. In fact, polyphenols show a low bioavailability due to several factors: interaction with the food matrix, the metabolic processes mediated by the liver (phase I and II metabolism), intestine and microbiota. On the other hand, the biological activities of phenol compounds may be mediated by their metabolites, which are produced in vivo, and recent studies have confirmed that these molecules may have antioxidant and anti-phlogistic properties. This review discusses the studies performed in vivo, which consider the polyphenol bioavailability and their different food sources. Factors influencing the biological effects of the main classes of polyphenols are also considered.

Keywords: polyphenols; bioavailability; beneficial effects; human studies

1. Introduction

It is known that an appropriate diet and lifestyle are essential for preserving well-being and preventing illnesses. Due to their abundance in foods derived from plants (e.g., vegetables, fruits, and beverages) and their potential antioxidant activity, polyphenols have been considerably studied in the past years as adjuvants in attenuating the risk factors for disabling diseases (mainly cardiovascular disease (CVD), diabetes, cancer, and cognitive disorders) [1]. Polyphenols are phenylpropanoids synthetized by plants as secondary metabolites, in adverse situations, such as in the presence of pathogens or under adverse climatic conditions. More than 8000 phenolic molecules have been identified, which must contain at least one aromatic nucleus and one or more -OH groups [2]. Polyphenols are commonly categorized as flavonoids, characterized by a C6-C3-C6 structure, and non-flavonoids. Flavonoids generally present in foods are anthocyanins, flavonols, flavan-3-ols, flavones, isoflavones, flavanones, and stilbenes (Figure 1).

Most flavonoids found in foods are conjugated with sugars, acids, or alcohols. Non-flavonoids include phenolic acids, in particular, hydroxybenzoic acids (i.e., vanillic and gallic acids), and cinnamic acids (i.e., ferulic and caffeic acids). All of these molecules have proven to have biological activities [3,4]; however, most of them have been shown using in vitro models and pure compounds, where the metabolism and

matrix effect were not taken into account. This is due to the fact that in vitro approaches enable the specific mechanisms of action of each molecule/group of molecules to be identified, with relatively low costs. Unfortunately, in vitro studies do not take into account the metabolic transformations and physiological concentrations [5].

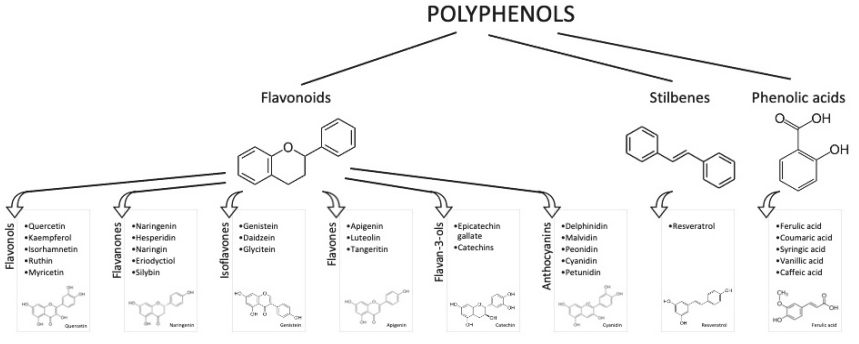

Figure 1. Polyphenol classes and chemical structures of some of their main compounds.

As regards in vivo studies, although several epidemiological and clinical studies have evaluated the polyphenol intake, several of them present several limitations, such as a low number of participants, no controls, the use of different methodologies to assess dietary habits, heterogeneous types of database to determine the consumption, etc. Despite the fact that other large and well-designed studies (for example, the PREDIMED study) have shown that the Mediterranean diet, characterized by foods rich in polyphenols, is associated with a reduction in cardiovascular risk and a better cognitive function in the elderly, strong evidence supporting its effects on human health is still not clear [6]. This is also due to the scarcity of knowledge regarding polyphenol bioavailability, together with the difficulty in determining which specific molecule is involved in the biological effect when different phenol compounds are present concomitantly. To date, only cocoa and extra virgin olive oil have thus received the approval of a health claim related to the content in phenol compounds.

Lastly, besides the polyphenol content in foods, in order to establish a correlation between the bioavailability and health effects, their mean intake in humans has to be considered. In a recent systematic literature revision, including more than 90 human studies, the polyphenol intake was estimated according to different dietary habits. Total polyphenol intake for the general population (inclusive of young people, adults, and the elderly) was estimated to be 0.9 g per day, where the main dietary founts were coffee, tea, wine (especially red wine), fruits, and vegetables. Total flavonoids and specific subclasses (flavonols, anthocyanidins, proanthocyanidins, flavan-3-ols, flavones, and flavanones) were associated with reduced CVD, diabetes mellitus (T2D), and mortality for all causes [7]. However, a correlation with the bioavailability of these compounds was not evaluated.

The goal of the present review was to assess the human bioavailability of the main classes of polyphenols, taking into account their food sources and the main factors affecting their in vivo accessibility.

2. Materials and Methods

The most important life-science databases of references and abstracts (PubMed, MEDLINE, Embase, and CAB-Abstract) were methodically analyzed (from database beginning to October 2020), using the following terms: “polyphenols”, “anthocyanins”, “flavanols”, “flavonols”, “flavans”, “stilbenes”,

"flavanones" in combination with "bioavailability", "disease", "health" and refining the results for "human studies" AND "controlled trials".

A search by title and abstract led to the collection of 98 relevant publications on the association between polyphenols and bioavailability. By removing duplicates and non-relevant papers (papers investigating the effects of polyphenols without considering bioavailability data, studies performed on animals, or using in vitro models), in total, 37 publications were included in this review.

3. Results and Discussion

3.1. Anthocyanins

Anthocyanins are pigments soluble in water contributing to the red, violet, and blue colors in flowers and fruits. At low pH, the anthocyanin chemical structure presents a positive charge at the oxygen atom of the C-ring, called the flavylium ion, and appears as red pigments. Anthocyanins are classified on the basis of the number and position of -OH groups on the flavonoid molecule. To date, more than 600 anthocyanin compounds have been identified [2]. Of these, the glycosylated form of cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin are the most abundant. The most common sugar in anthocyanidin glycosides is glucose; however, rhamnose, galactose, and rutinose may also be present. The sugar group can be acylated, generally at C3 position, by aromatic acids such as hydroxycinnamic acids (ferulic, caffeic, and coumaric).

Anthocyanins are among the polyphenol compounds with the highest concentration in foods, with an average concentration of 115 $\pm$ 259 mg 100 g^{-1} [8]. The richest sources of anthocyanins (as glycosides) are black elderberries (1316 mg 100 g^{-1}), black chokeberry (878 mg 100 g^{-1}), and black currant (595 mg 100 g^{-1}). Apart from red fruits, important sources of anthocyanins are represented by red wine, colored beans, and vegetables such as red oranges, red lettuce, or red onions. Like other flavonoids, the mean daily intake of anthocyanins can vary among countries, depending on the nutritional habits and cultural differences. It has been calculated that the daily intake of anthocyanins is between 6.8 mg per day in Brazil (where the most important dietary sources are citrus and tropical fruits) and Australia, and 133 mg per day in Italy (the main dietary sources are seasonal fruits, citrus fruits, leafy vegetables, and wine) [9–11].

Anthocyanin bioavailability is extremely low: only about 1–2% maintain their original structure after ingestion [12]. Anthocyanins exist in different chemical structures depending on the pH. In the stomach, at pH 1.5–3, the main chemical forms are flavylium cations, while in the intestinal environment, the carbinol forms predominate, with lower absorption. In addition, other biotransformation steps occur during gastrointestinal digestion, such as phase II metabolism processes (glucuronidation, sulphation, and methylation), enzymatic and microbiota catabolism [12]. These lead to several chemical compounds, namely anthocyanin glucuronides, phenolic acids (ferulic acid, caffeic acid, vanillic acid, gallic acid, protocatechuic acid, syringic acid, and 4-hydroxybenzoic acid), and aldehydes (phloroglucinaldehyde and phloroglucinaldehyde) [12]. Nevertheless, there is a remarkable inter- and intra-variability in the bioavailability of anthocyanins, due to several factors, such as the food matrix or technological/processing conditions, enzymatic patterns, and microbiota composition. Only a few studies have evaluated a correlation among the results obtained and the bioavailability data. Table 1 summarizes the human trials where the health properties of anthocyanins are correlated with bioavailability studies. The literature data show that the anthocyanin daily doses used in the clinical trials ranged between 2.1 and 94.47 mg. These amounts were generally provided by food (blackcurrant and orange juice); and only the study by Xie et al. (2017) used a food supplement [13]. When anthocyanins were consumed in food, they did not affect the biomarkers investigated, mainly associated with the cardiovascular function, such as the oxidative status, inflammation and vascular reactivity. Only in the study by Xie et al., (2017) 500 mg/day of Aronia

extract (*Aronia melanocarpa*, also known as black chokeberry), containing 45.1 mg anthocyanins, 35.7 mg hydroxycinnamic acids, and 41.9 mg proanthocyanidins, improved the total plasma cholesterol and LDL (Low-Density Lipoproteins) receptor in peripheral blood mononuclear cells [13]. This positive effect was due to the duration of the study (12 weeks vs acute consumption or a maximum of four weeks in the other studies) and by the synergistic effect of other phenol compounds present in the extract (hydrocynnamic acids and proanthocyanidins). These results are also supported by the literature data, where the chronic consumption, from six weeks to two months, of Aronia berry extracts (255–300 mg/day) decreased several biomarkers of cardiometabolic diseases, due to the high Aronia polyphenol content [14,15]. In addition, the studies were generally performed on healthy subjects, thus making it more difficult to measure evident effects on the health status.

Regarding the bioavailability data, the amounts of anthocyanins found in plasma or urine were generally low, which was correlated with the dose taken and the kind of food provided in the studies. Jin et al. (2011) hypothesized that blackcurrant juice did not ameliorate the vascular reactivity in healthy subjects, due to the low levels of anthocyanins in the juice (20%) [16]. This observation has been supported by other authors, who reported that a consistent elevation of plasma anthocyanins and, as a consequence, significative health effects, can be observed only when anthocyanins are consumed at pharmacological levels (500–1500 mg/day) [17,18]. It is also noticeable that anthocyanin compounds are affected by high instability and susceptibility to degradation, particularly at the gastrointestinal level [19]. In addition, the short periods of supplementation or juice ingestion (maximum 12 weeks) were also found to influence the lack of a significant reduction in oxidative status and CVD biomarkers. In urine, the percentage of anthocyanins varied between 0.009 ± 0.002 and $0.79 \pm 0.90\%$ of the dose taken, in line with the literature data [20,21]. Despite the great variability in anthocyanin food content, cyanidin and peonidin glucosides were generally considered the most available, since their metabolites were the only ones measurable in plasma or urine. In their clinical trial, Xie et al. (2017) speculated that the 8% reduction in fasting plasma total cholesterol after 12 weeks of supplementation with Aronia extract was also due to cyanidin methylated metabolite and 3-(4-hydroxyphenyl)propionic acid derived from microbiota fermentation [13]. However, the mechanism by which these metabolites affect lipid metabolism needs to be further explored. Despite the general lack of positive effects on CVD biomarkers, several trials have described positive effects of anthocyanins when included in the daily diet. For example, Wedick et al. (2012) reported that the anthocyanin intake of about 22.3 mg per day and an anthocyanin-rich fruit consumption ($\geq$5 times per week) was correlated with a minor risk of developing type-2 diabetes [22]. Cassidy et al. (2016) reported a decreased risk of myocardial infarction in normotensive patients > 65 years, when high levels of anthocyanins were consumed (>35 mg/day) [23]. Other studies reported positive effects of food supplements containing anthocyanins on ocular function, showing encouraging results in relation to glaucoma and in the reduction of retinal oxidative stress due to aging. However, in these studies, anthocyanin bioavailability was not considered. Manach et al. (2005) partially explained the discrepancies in the positive effects of anthocyanin despite their low anthocyanin bioavailability, by considering the following: (1) the possible presence of anthocyanin metabolites not measured in biological samples, such as microbiota metabolites; (2) the instability of anthocyanin metabolites (glucuronides and sulfates), which can extensively degrade in frozen urine, during storage [20].

Table 1. Anthocyanin's beneficial effects and bioavailability in human subjects.

Cohort and Study Details	Anthocyanin Intake	Aim	Bioavailability Data	Outcome	Reference
49 healthy adult former smokers (25 M; 24 F) (mean age 35 ± 2.8 years) Duration: 12 weeks Randomized controlled trial	• Treatment group (n = 25): 500 mg *Aronia melanocarpa* extract (9.02% anthocyanins, 7.14% hydroxycinnamic acids and 8.38% proanthocyanidins). • Placebo group (n = 24): 500 mg rice powder with 0.2% beet juice concentrate.	Modulation plasma lipids (change in LDL cholesterol was the primary outcome), blood pressure, biomarkers of inflammation and oxidative stress, lipid transport genes of peripheral blood mononuclear cells.	Overnight urinary anthocyanins were significantly higher in the treatment group vs. placebo group (0.332 ± 0.136 vs. 0.051 ± 0.022 mg mg^{-1} creatinine). Urinary peonidin-3-galactoside was 0.0062 ± 0.0026 mg mg^{-1} creatinine in the treatment group vs. 0.0008 ± 0.0005 mg mg^{-1} creatinine in placebo group ($p < 0.05$). The excretion of the other polyphenols was not significantly affected.	After 12 weeks, Aronia consumption compared with the placebo group showed: ↓ 8% fasting plasma total cholesterol (p = 0.0140), ↓ 11% LDL cholesterol (p = 0.02), ↓ LDL receptor protein in peripheral blood mononuclear cells (p = 0.0036).	[13]
20 Healthy subjects (UK) (9 M; 11 F) mean age (44.55 ± 13.34 years) Duration: acute consumption Randomized, double-blind, placebo-controlled crossover study	250 mL of either a blackcurrant juice drink (20% of anthocyanins) or the control drink. Sample collection after consumption: • Blood: periodically up to 480 min; • Urine: every 2 h and at 24 h.	To measure vascular reactivity at 120 min after juice consumption.	The urinary percentage anthocyanins excreted after 120 min was 0.021 ± 0.003% and 0.009 ± 0.002% of the dietary intake of delphinidin glycosides and cyanidin glycosides, respectively.	No significant effects on vascular reactivity were found. An increase in plasmatic vitamin C was observed (p = 0.006).	[16]
15 subjects with coronary artery disease (13 M; 2 F) (mean age: 62 ± 8 years) Duration: acute consumption Pilot study	480 mL cranberry juice (54% juice; 835 mg total polyphenols; 94.47 mg anthocyanins). Sample collection after consumption: • Plasma and urine: after 4 h.	To evaluate plasma redox capacity.	Plasma concentrations ranged between 0.56 and 4.64 nmol L^{-1}. Total urinary anthocyanins were 0.79 ± 0.90% of the amount taken. Cyanidin-glucoside and peonidin-glucoside were the most available (0.007 ± 0.004% and 0.029 ± 0.059% of the dose delivered).	Anthocyanin plasmatic concentrations were not able to reduce oxidative stress.	[24]
20 healthy females (mean age: 27.8 ± 7 years) Randomized controlled trial Duration: 2 weeks	• Treatment group (n = 11) 750 mL per day (3 × 250 mL) of cranberry juice (total anthocyanins = 2.80 ± 0.19 mg L^{-1}; where 29.2% peonidin galactoside, 26.1% cyanidin arabinoside, 21.7% cyanidin galactoside, 17.5% peonidin arabinoside, 4.1% peonidin glucoside, and 1.4% cyanidin glucoside). • Placebo group (n = 9): natural mineral water with strawberry flavor.	Plasma antioxidant activity and biomarkers of oxidative stress (total phenol concentrations, reduced glutathione levels (GSH) and plasma free radical trapping capacity (FRAP)). Activity of blood's antioxidant enzymes (SOD, catalase and GSH-Px). Urinary excretion of MDA and 8-oxo-deoxyguanosine (as marker of DNA damage).	Neither anthocyanins nor catechins were detectable in plasma samples isolated from both groups, only vitamin C increased significantly ($p < 0.01$) in the cranberry juice group. Further, catechins were not detectable in the urine samples.	Hcy, TC, TG, HDL, and LDL were unchanged. The antioxidant potential of the plasma, GSH-Px, catalase and SOD activities, MDA and 8-oxo-deoxyguanosine levels were not significantly different in both groups.	[25]

Table 1. *Cont.*

Cohort and Study Details	Anthocyanin Intake	Aim	Bioavailability Data	Outcome	Reference
18 healthy subjects (10 M; 8 F) (25–47 years) Duration: 4 weeks Randomized crossover study	1 L per day of either blood orange juice (OJ) (from Moro, Tarocco, and Sanguinello varieties) or blond OJ that contains no anthocyanins (from Valencia, navel, and Belladonna varieties). Blood orange juice contained: 53.09 ± 5.31 mg L^{-1} total anthocyanins, 3.96 ± 0.20 mg L^{-1} delphinidin-3-glucoside, 25.79 ± 1.17 mg L^{-1} cyandin-3-glucoside, 17.88 ± 0.95 mg L^{-1} cyanidin-3-(6-malonylglucoside).	Potential effects on cell markers of platelet and leukocyte activation (P-selectin, PAC-1, leukocyte activation markers CD11b) due to anthocyanins absorption after daily supplementation with blood OJ for 1 month.	Mean levels of anthocyanins (11.47 ± 5.63 nmol L^{-1}) significantly differed from baseline in 24-h urinary excretion. Anthocyanins levels remained substantially unchanged until the end of treatment ($p = 0.1$).	The anthocyanin plasma levels reached were insufficient to significantly modify cell markers of platelet and leukocyte activation and interaction. Urinary excretions of anthocyanins considered showed a significant increase after blood OJ consumption ($p < 0.05$).	[26]
16 healthy females (20–27 years) Duration: 3 weeks Randomized crossover study	• Group A ($n = 8$) - Standardized diet without orange juice; - Wash out period; - Standardized diet with 600 mL/day of blood orange juice. • Group B ($n = 8$) - Standardized diet with 600 mL/day of blood orange juice; - Wash-out period; - Standardized diet without orange juice. Each period was of 21 days. 100 mL of orange juice contained: 75.2 mg vitamin C, 67 µg of β-cryptoxanthin, 20 µg of lutein, 18 µg of zeaxanthin, 17 µg of lycopene, 10 µg of β-carotene, and 8 µg of α-carotene, 3.5 mg cyanidin-3-glucoside, 1.2 mg cyanidin-3-glucoside-6″-malonyl.	To evaluate the effect on plasma antioxidant concentrations and on markers of lipid peroxidation malondialdehyde (MDA) and 11-dehydro-tromboxane 2 (TXB2)	Cyanidin 3-glucoside mean plasma concentration was, after the washout period, about 0.6 nmol L^{-1} and increased from 0.56 nmol L^{-1} to ∼8 nmol L^{-1} after 3 weeks of blood orange juice intake ($p < 0.05$). Both the aglycone and the cyanidin-3-glucoside-6″-malonyl were not detected in plasma.	Plasma antioxidant capacity did not increase after the 3 weeks of juice intake. The daily intake of orange juice did not affect the biomarkers of lipid oxidation malondialdehyde (MDA), and 11-dehydro-TXB2.	[27]

LDL, Low Density Lipoproteins; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; PAC-1, procaspase-activating compound-1; SOD, superoxide dismutase; OJ, orange juice; Hcy, Homocysteine; TG, triglycerides.

3.2. Flavanols

Flavanol compounds are included in a wide of range of foods. The main sources of flavanols are cocoa (3411 mg 100 g^{-1}) and dark chocolate (1590 mg 100 g^{-1}). Berries are also an important source of flavanols, containing 659, 330, and 139 mg 100 g^{-1} for black chokeberry, blueberry, and blackcurrant, respectively. Strawberry (148 mg 100 g^{-1}) and apple (111 mg 100 g^{-1}), as well as hazelnut, pecan nut, pistachio, and almonds (181–496 mg 100 g^{-1}), are other important sources. Black tea, green tea, and red wine contain high levels of flavanols, in particular, catechins and proanthocyanidin dimers, with estimated mean amounts of 18–50 mg in the daily diet [20].

However, the content of some flavanols is often underestimated, since generally the methods used (e.g., HPLC) only evaluate monomers and proanthocyanidin dimers and trimers. The intake of flavanols in the European Union, according to the EFSA (European Food Safety Authority) Comprehensive European Food Consumption Database, and the average intake of flavan-3-ol monomers, theaflavins, and proanthocyanidins ranges from 181 mg per day (Czech Republic) to 793 mg per day (Ireland) [28].

The highest intakes of flavan-3-ols (monomers) and theaflavins were detected in Ireland (191 and 505 mg per day, respectively) and the lowest in Spain (24 and 9 mg per day, respectively). On the other hand, the highest daily intake of proanthocyanidins (PA) was found in Spain (175 mg per day) and lowest in The Netherlands (96 mg per day). The most important sources were tea (62%), pome fruits (such as apples and pears) (11%), berries (3%), and cocoa derivatives (3%). Tea was the principal contributor to monomer intake (75%), followed by pome fruits (6%). In addition, pome fruits were the most important source of proanthocyanidins (28%). Table 2 reports the clinical trials where the flavanol intakes and their bioavailability were correlated with beneficial effects. Flavanol intake ranged between 28.3 to 907.5 mg/day. These amounts were chosen on the basis of the mean dietary intake of flavonosl among the population included in the studies, and thei consumption with food (enriched chocolate or coffee). Participants in the studies were generally healthy or had stage 1 hypertension without concomitant risk factors.

Dower et al. (2016) compared the chocolate consumption (containing 150 mg epicatechin, EC) with pure epicatechin supplementation (100 mg) [29]. The length of treatment varied between acute consumption to 18 weeks. The principal outcomes investigated were associated with cardiovascular function (e.g., blood pressure, flow-mediated dilation, and platelet function) and antioxidant activity (e.g., LDL oxidation or plasma 8-isoprostane). A significant improvement in cardiovascular biomarkers was also generally observed for the consumption of low levels of flavanols (28.3 mg/day). A significant improvement in cardiovascular biomarkers was also generally observed for the consumption of low levels of flavanols (28.3 mg/day), despite the EFSA highlighting of a cause-effect correlation between cocoa consumption and endothelium vasodilation for a daily intake of 200 mg [30]. In contrast, only a few studies have measured notable effects on oxidative stress, suggesting that other mechanisms can be involved in the improvement of cardiovascular function [31,32].

According to Taubert et al. (2007), the positive results were generally associated with the monomers epicatechin/catechin and the dimers procyanidin B2/procyanidin B2 gallate, which were the only monomers dosed in plasma, at a concentration ranging between 0.14 ($\pm$0.06) ng/mL of procyanidin B2 gallate and 3.63 ($\pm$1.02) ng/mL (from 1 to 6 h after consumption) [32]. These amounts reduced blood pressure by 1.9 ($\pm$1) mmHg in patients with stage 1 hypertension probably due to the "chronic" increase in endothelium NO production. Similar effects were observed in other studies where higher levels of flavan-3-ols were used, due to the shorter period of intake. Interestingly, in the clinical trial by Dower et al. (2016), the positive effect of epicatechin (EC) on flow-mediated dilatation (FMD) from dark chocolate was not observed for pure epicatechin administered in association with white chocolate, suggesting a significant impact of the food matrix and sugar content on epicatechin bioavailability [29].

Urinary flavan-3-ols were measured only by Ostertag et al. (2013), who described a maximum catechin excretion of 13.4 mmol/mol creatinine 2–6 h after dark chocolate intake (containing 907.5 mg total catechins) [33]. As regards platelet function, flavan-3-ols affected platelet aggregation 120 min after intake, when a peak plasma concentration was obtained. However, bleeding time was affected only after 360 min, when the colonic metabolites kicked in Reference [33]. Interestingly, a gender-specific modulation of platelet aggregation reduction was also observed, probably due the formation of larger platelet aggregates after adenosine diphosphate (ADP) stimulation in women. It should also be noted that flavan-3-ol bioavailability varies markedly among the different subclasses. Manach et al. (2005) reported that galloylated catechins are poorly absorbed, explaining the higher bioavailability and the activity of epicatechin described in the clinical trials [20]. In addition, epicatechin glucuronide and sulfate metabolites, together with valerolactone microbial derivatives—not measured in the clinical trials included in this review—account for 6–39% of the ingested epicatechin, thus prolonging its biological effects [34].

As regards procyanidins, these compounds have a reduced bioavailability, which is about 100-fold lower than monomers. The biological effects are generally due to the monomers formed after gastric degradation, which can be rapidly absorbed in the gut. In addition, the gut microbiota is responsible for the metabolite formation (m-hydroxyphenylpropionic acid, m-hydroxyphenylacetic acid, phenylpropionic acid, phenylacetic acid, hydroxyphenylvaleric acid, and benzoic acid among others), which could also be responsible for various biological effects [20].

Table 2. Flavanols' beneficial effects and bioavailability in human subjects.

Cohort and Study Details	Flavanol Intake	Aim	Bioavailability Data	Outcome	Reference
20 healthy men (40–80 years) Duration: acute consumption randomized, placebo controlled, crossover study	Subjects consumed the following: • Dark chocolate (DC) (70 g, containing 0.15 g EC) associated with 2 capsules of placebo; • Pure EC (0.050 g, purity 96.2%) (total EC = 0.100 g) associated with white chocolate (WC) (75 g); • 2 capsules of placebo with 75 g of WC (without EC).	To study the effect of epicatechin from different matrices (cocoa and supplement) on vascular function (FMD and AIx).	DC intake determined an Area Under the Curve of 16.2 μM (per 0.100 g of EC) that was not significantly different from Area Under the Curve calculated for pure EC ($p = 0.14$).	Pure EC didn't ameliorate Flow Mediated Dilatation (+0.75%; $p = 0.10$) or Augmentation Index (2.2%; $p = 0.23$) respect to placebo. DC significantly ameliorated Flow Mediated Dilatation (+0.96%; $p = 0.04$) and Augmentation Index (4.6%; $p = 0.02$). Amelioration of FMD (+0.21%; $p = 0.65$) or Aix (–2.4%; $p = 0.20$) was not different after pure EC and DC intake. EC bioavailability was not different after pure EC and DC ($p = 0.14$).	[29]
21 healthy subjects (11 M; 11 F) (mean age: 32.2 ± 3.1 years) Duration: 2 weeks Randomized, double-blind, controlled with placebo study	The following treatments were assigned: • High-flavonoid chocolate intake (0.213 g PC, 0.046 g EC) per day; • Low-flavonoid DC (46 g) per day.	To evaluate the impact of DC enriched with flavonoids on FMD, blood pressure, markers of oxidative stress (LDL-ox, total antioxidant power, 8-isoprostane levels and plasmatic lipid concentrations).	Plasmatic EC levels were significantly higher at two weeks after high-flavonoid DC consumption (204.4 ± 18.5 nmol L^{-1}, $p <$ or =0.001) than after low-flavonoid DC intake (17.5 ± 9 nmol L^{-1}, $p = 0.99$).	The intake of chocolate containing high levels of flavonoids ameliorated FMD (average change = 1.3 ± 0.7%) respect to the groups consuming chocolate with low levels of flavonoids (mean change = −0.96 ± 0.5%) ($p = 0.024$). LDL-ox, antioxidant power, 8-isoprostanes, blood pressure, lipid biomarkers were not significantly in the two groups.	[31]

Table 2. *Cont.*

Cohort and Study Details	Flavanol Intake	Aim	Bioavailability Data	Outcome	Reference
44 adults (20 M; 24 F, 20) (Age: 63.6 ± 4.8 years) with not-treated hypertension (at stage 1) without other diseases Duration: 18 weeks Randomized study	Treated subjects (n = 22): 6.3 g dark chocolate per day providing 30 mg of polyphenols per day (1.4 mg gallic acid, 1.7 mg catechin, 5.1 mg epicatechin, 0.3 mg epicatechin-gallate, 6.8 mg procyanidin dimer, 1.8 procyanidin dimer-gallate, 5.3 mg procyanidin trimer, 1.0 mg procyanidin trimer-gallate, 3.7 mg procyanidin tetramer, 2.6 mg procyanidin pentamer, <0.05 mg flavonols, 3.1 mg caffeine, 26.4 mg theobromine) Control group (n = 22) polyphenol-free white chocolate.	To evaluate the change in blood pressure. Secondary objectives were the assessment of nitric oxide and oxidative stress plasmatic biomarkers (S-nitrosoglutathione and 8-isoprostane, respectively).	Only catechin, epicatechin and the dimers procyanidin B2 and procyanidin B2 gallate were measured in plasma and didn't change after 18 weeks. Pharmacokinetic data were: Epicatechin: AUC from 761 (±210) ng mL^{-1} × min on day 1 to 774 (±253) ng mL^{-1} × min (p = 0.82) at 18 weeks; Cmax from 3.63 (±1.02) ng/mL on day 1 to 3.58 (±0.92) ng mL^{-1} (p = 0.78) at 18 weeks; Tmax from 77 (±4) min on day 1 to 81 (±6) min at 18 weeks (p = 0.70); T1/2 from 54 (±3) min on day 1 to 56 (±5) min (p = 0.79) at 18 weeks. Catechin: AUC from 234 (±61) (day 1) to 228 (±56) ng mL^{-1} × min (p = 0.77); Cmax from 1.12 (±0.31) ng mL^{-1} on day 1 to 1.01 (±0.26) ng mL^{-1} (p = 0.58) at 18 weeks; Tmax from 78 (±9) min on day 1 to 82 (±6) min at 18 weeks (p = 0.69); T1/2 from 54 (±6) min on day 1 to 58 (±7) min (p = 0.68) at 18 weeks. Procyanidin B2: AUC from 99 (±30) ng mL^{-1} × min on day 1 to 102 (±32) ng mL^{-1} × min (p = 0.90); Cmax from 0.45 (±0.15) ng mL^{-1} on day 1 to 0.43 (±0.14) ng mL^{-1} (p = 0.94) at 18 weeks; Tmax from 81 (±8) min on day 1 to 86 (±9) min at 18 weeks (p = 0.62); T1/2 from 56 (±6) min on day 1 to 57 (±5) min (p = 0.91) at 18 weeks; procyanidin B2 gallate: AUC from 33 (±14) ng mL^{-1} × min on day 1 to 33 (±13) ng mL^{-1} × min (p = 0.91); Cmax from 0.14 (±0.06) ng mL^{-1}- day 1 to 0.14 (±0.06) ng mL^{-1} (p = 0.98) at 18 weeks; Tmax from 89 (±10) min on day 1 to 85 (±8) min at 18 weeks (p = 0.72); T1/2 from 62 (±7) min on day 1 to 59 (±6) min (p = 0.76) at 18 weeks.	At the end of the study, the treatment with dark chocolate educed mean systolic blood pressure by 2.9 (±1.6) mm Hg (p < 0.001) and diastolic blood pressure by 1.9 (±1) mm Hg (p < 0.001) but 8-isoprostane levels didn't change. Hypertension prevalence was reduced from 86% to 68%. S-nitrosoglutathione levels (increased by 0.23 nmol L^{-1} (±0.12) (p < 0.001).	[32]

Table 2. *Cont.*

Cohort and Study Details	Flavanol Intake	Aim	Bioavailability Data	Outcome	Reference
42 healthy subjects (26 M; 16 F) (mean age 41 ± 2.0 years) Duration: acute consumption Observer-blinded randomized-controlled crossover acute intervention trial	Subjects received 60 g of dark chocolate enriched with flavan-3-ols (with 400 mL water), 60 g of a "standard" dark chocolate or 60 g of white chocolate (14 days wash out period was used between treatments). Sixty grams of chocolate enriched with flavan-3-ols contained: 0.257 (±1.06) g epicatechin, 53.6 (±0.27) mg catechin, 0.198 (±1.22) g dimer B2, 0.168 (±1.42) g trimers, 0.105 ± (12.75) g tetramers, 0.125 ± (6.04) g pentamers (total flavonoids = 907.4 ± 22.75 mg). Sixty grams of "standard" dark chocolate contained: 84.1 (±0.67) mg epicatechin, 25.8 (±1.02) mg catechin, 74.4 (±0.76) mg dimer B2, 47 (±2.23) mg trimers, 32.1 (±4.4) mg tetramers, 0.119 (±39.5) g pentamers (total flavonoids: 382.3 ± 45.2 mg). White chocolate = ND.	To evaluate if flavan-3-ol-enriched dark chocolate could influence markers of platelet status (adenosine diphosphate-induced platelet aggregation, expression of P-selectin, thrombin receptor-activating peptide-induced platelet aggregation and thrombin receptor-activating peptide-induced fibrinogen binding, collagen/epinephrine-induced bleeding time).	Plasmatic levels of total C/EC significantly increased 120 min after flavan-3-oil enriched or "standard "dark chocolate in comparison with white chocolate ($p < 0.001$) (Cmax = 1.20 µmol L^{-1} 120 min after consumption of dark chocolate enriched with flavan3-ols) and decreased after 6 h. Urinary levels of total catechins increased 120 and 360 min after consumption of enriched dark or "standard" dark chocolate in comparison with white chocolate ($p < 0.001$), reaching a peak concentration of 13.4 mmol mol^{-1} creatinine. Comparable results were observed for procyanidin dimer B 2 ($p < 0.001$), reaching a peak concentration at 57 µmol mol^{-1} creatinine at 360 min. The peak of flavan-3-ol concentrations was higher in biological fluids of women ($p = 0.047$).	Enriched dark and white chocolate ameliorated different biomarkers of platelet status (adenosine diphosphate-induced platelet aggregation, P-selectin expression, thrombin receptor-activating peptide-induced platelet aggregation and thrombin receptor-activating peptide-induced fibrinogen binding) following a gender-specific fashion.	[33]
18 healthy male subjects (mean age: 36 ± 10 years) Duration: acute consumption Observational study	Subjects consumed 50 g of 90% cocoa chocolate (containing 7.5 g total polyphenols expressed as GA equivalents) within 5 min.	To investigate the impact of dark chocolate on platelet status and the correlation with flavanols metabolites.	After 4 h from the ingestion, epicatechin metabolites concentration in plasma significantly increased (epicatechin glucuronide, sulfate, methyl-epicatechin-sulfate) ($p < 0.05$).	An inhibitory effect of cocoa was observed on platelet aggregation and adhesion caused by ADP/collagen 4 h after cocoa intake (98.5 ± 13.0 respect to 114.5 ± 22 s, $p < 0.001$) was noticed, while the closure time of collagen/epinephrine didn't change (128.5 ± 27.0 vs. 122.5 ± 34. s, $p = 0.33$), probably associated with EC metabolites.	[35]

ADP, Adenosine diphosphate; AUC, area under the curve; DC, Dark Chocolate; EC, epicatechin; FMD, flow-mediated dilatation; GA, gallic acid; Aix, Augmentation Index; PC, procyanidins; LDL-ox, oxidated LDL.

3.3. Flavonols

Fruits, vegetables, and some beverages, like tea and red wine, are the main source of flavonols, for which the intake is estimated between 18 (USA) and 58 mg (Japan) per day [28]. However, these intake levels generally refer only to the three main flavonols, namely quercetin, myricetin, and kaempferol. In fruits and vegetables, the highest quercetin content is found in cranberry (149 mg/100 g) and onions (65 mg 100 g^{-1}), while in green tea and red wine, the mean contents are 2.5 and 1.6 mg/100 mL, respectively [36]. Eleven studies have explored the different health effects of flavonol intake, mainly on the reduction in CVD risk factors (homocysteine and LDL-oxidation levels, homocysteine, plasmatic High Density Lipoproteins (HDL) and LDL cholesterol, blood pressure, NO production, and platelet aggregation), inflammatory biomarkers (C-reactive protein and endothelin-1 expression), and antioxidant activity (e.g., excretion of urinary F2-isoprostanes and plasma malondialdehyde (MDA)) (Table 3). One study also investigated the effect of an enzymatically modified quercetin on cognitive function [37]. These effects have generally been measured in healthy subjects, apart from three studies involving subjects at cardiovascular risk [37–39].

In most studies, flavonols were administered by extracts, given alone or mixed with food; only in three studies they were provided using food preparations (onion soup or cake) [40–42]. Flavonol intake ranged between 16.7 to 400 mg/day and included mainly quercetin and its derivatives, isorhamnetin and kaempferol. The data in Table 3 show that cardiovascular parameters, as well as oxidative stress biomarkers, were generally not affected by flavonols. Suomela et al. (2006) and Larmo et al. (2009) used a sea buckthorn extract (*Hippophae rhamnoides* L.) as the source of flavonols (78 and 16.7 mg per day, respectively), administered with meals [38,43]. Both flavonol intakes failed to reduce oxidative stress, total and LDL cholesterol, or C reactive protein (CRP) concentration. Sea buckthorn was administered because of its traditional use in Eastern countries and the encouraging results from clinical and epidemiological studies reporting a reduction in cardiovascular risk factors [44]. The authors explained the different outcomes of their studies, using a moderate berry dose, similar to the average consumption in the daily diet and to the dosage (3–9 g) prescribed by the Chinese Pharmacopoeia for ameliorating hematic circulation [45]. These amounts were lower than those used in other trials (600 mg sea buckthorn flavonols), where positive results were reported [46].

Table 3. Flavonols' beneficial effects and bioavailability in human subjects.

Cohort and Study Details	Flavonol Intake	Aim	Bioavailability Data	Outcome	Reference
25 participants (12 M; 13 F) (mean age: 64.1 ± 6.3 years) with at least one CVD risk factor (sBP 120–160 mmHg, FPG 5.6–6.5 mM, total cholesterol 5–8 mM or a waist circumference > 94 cm for men or >80 cm for women) Duration: acute consumption Randomized, controlled crossover trial	Treatment group: - 4.89 mg/kg bw/day of EMIQ® (Enzymatically Modified IsoQuercitrin), - half teaspoon of maltodextrin, - one and half tablespoons of Cottee's Raspberry flavored cordial. Placebo group: - half teaspoon of maltodextrin, - one and half tablespoons of Cottee's Raspberry flavored cordial, The treatments were given in 250 mL of water.	To evaluate if FMD, BP, and cognitive function improve whether an acute intake of EMIQ® was administered.	After 3 h from the consumption of EMIQ®, quercetin metabolites concentration was significantly higher in plasma respect placebo group (quercetin aglycone 144.9 ± 12.3 nM vs. 12.6 ± 12.3 nM; and isorhamnetin 245.5 ± 16.5 nM vs. 41.7 ± 16.5 nM) ($p < 0.001$).	EMIQ® significantly affected FMD compared with the placebo ($p = 0.025$).	[37]
14 healthy males (46.6 ± 5.6 years) with a slightly elevated total cholesterol level (5.3–7.2 mmol/L) Duration: 4 weeks Double-blind, placebo-controlled, crossover study	• Group 1: daily consumption of 185 g of an oatmeal porridge supplemented with a flavonols extract of sea buckthorn (*Hippopae rhamnoides* L.) • Group 2: consumption of control porridge without flavonols. 0.4 g of extract, added to the porridge, contained: 78 mg of total flavonol aglycones, of which 70% isorhamnetin, 26% quercetin, and 4% kaempferol.	To evaluate the effects on CRP, conjugated dienes and oxidized LDL, homocysteine, and paraoxonase activity (potential risk factors of CVD) of a flavonols extract of sea buckthorn.	Flavonols were mainly present as glucuronide and sulfate metabolites in plasma fluid. When was intake porridge added with flavonols extract, AUC was significantly higher for kaempferol and isorhamnetin ($p < 0.05$).	The flavonols ingested did not significantly affect the following: - Oxidized LDL; - CRP; - Homocysteine levels; - Plasma antioxidant potential; - Paraoxonase activity.	[38]
9 overweight/obese men ($n = 4$) and post-menopausal women ($n = 5$) (mean age = 55.9 ± 2.1 years) Duration: acute consumption Randomized, crossover study	Subjects ingested quercetin aglycone (1095 mg) with 3 types of standardized breakfast: - Fat-free (<0.5 g); - Low-fat (4.0 g); - High-fat (15.4 g).	To verify whether dietary fat improve quercetin and its metabolites bioavailability in adults with high CVD risk.	During the high-fat breakfast, compared to the fat-free trial: - Plasma quercetin: ↑ 45% Cmax; ↑ 32% AUC (0–24 h); - plasma isorhamnetin: ↑ 40% Cmax; ↑ 19% AUC (0–24 h); - Plasma O-methyl-isorhamnetin: ↑ 46% Cmax; ↑ 43% AUC (0–24 h).	Dietary fat improved quercetin bioavailability by increasing its absorption leading to a possible dietary approach for reducing CVD risk.	[39]

Table 3. *Cont.*

Cohort and Study Details	Flavonol Intake	Aim	Bioavailability Data	Outcome	Reference
6 heathy subjects (4 M; 2 F) (mean age 34 ± 7 years) Duration: 1 day Randomized, double-blind, crossover study	Participants ingested either a high- or a low-quercetin soup (600 mL), made using 500 g of onions for portion. 1 L of low quercetin onion soup (LQS) contained 0.1 mg L^{-1} quercetin aglycone, 3.8 mg L^{-1} quercetin-4′-glucoside, 4.3 mg L^{-1} quercetin 3,4′-glucoside. 1 L of high quercetin onion soup (HQS) contained: 1.1 mg L^{-1} quercetin aglycone, 53.2 mg L^{-1} quercetin-4′-glucoside, 60.5 mg L^{-1} quercetin 3,4′-glucoside.	To investigate the possible inhibitory effects of quercetin ingestion from a dietary source on platelet function (collagen-stimulated platelet aggregation and collagen-stimulated tyrosine phosphorylation).	After HQS treatment, plasma levels of quercetin did the following: - Peaked at 2.59 ± 0.42 mmol L^{-1} ($p = 0.0001$); - AUC 911.61 ± 85·17 mmol L^{-1} per min ($p = 0.001$). Plasma levels of isorhamnetin peaked after 2 h at 0.119 ± 0.02 mmol L^{-1} (HQS) and 0.0133 ± 0.04 mmol L^{-1} (LQS) ($p = 0.0001$). Plasma levels of tamarixetin peaked after 2.5 h at 0.172 ± 0.035) mmol L^{-1} (HQS) and 0.0049 ± 0.001 mmol L^{-1} (LQS) ($p = 0.0001$).	HQS treatment inhibited the following: - Collagen-stimulated platelet aggregation (time-dependent); - Collagen-stimulated tyrosine phosphorylation ($p = 0.001$). The inhibition of tyrosine phosphorylation was correlated with AUC of quercetin after HQS intake.	[40]
36 healthy human subjects (16 M; 20 F) (mean age: 31.4 ± 7.7 years) Duration: 4 weeks Randomized crossover study	Treatment period: high flavonol (HF) diet based on daily consumption of 150 g onion cake (89.7 mg quercetin) + 300 mL black tea (1.4 mg quercetin). Control period: low flavonol (LF) period based on exclusion of flavonol and flavone foods and tea.	To determine the effect of dietary intake of quercetin from onions and black tea on oxidative damage to leukocytes DNA bases.	Plasma quercetin was <LOD (66.2 nmol L^{-1}) after the HF period and increased at 228.5 ± 34.7 nmol L^{-1} after HF period.	The concentrations of the products of oxidative damage to DNA bases did not differ significantly between the two dietary treatment periods for any of the products measured.	[41]
32 healthy subjects (mean age 30.4 ± 7.3 years) Duration: 4 weeks Randomized crossover study	Treatment period: high flavonol (HF) diet based on daily consumption of 150 g onion cake (89.7 mg quercetin) + 300 mL black tea (1.4 mg quercetin). Control period: low flavonol (LF) period based on exclusion of flavonol and flavone foods and tea.	To investigate the effects of a high-flavonoid (HF) diet on markers of oxidative stress (F2 -isoprostanes and malondialdehyde (MDA)-modified LDL) compared with a low-flavonoid (LF) diet.	After the HF treatment, plasma quercetin concentrations were significantly higher (221.6 ± 37.4 nmol L^{-1}) than after the LF treatment (compared with less than the LOD of 66.2 nmol L^{-1}).	There were no significant differences in plasma F2-isoprostane concentrations, and MDA–LDL between the HF and LF dietary treatments.	[42]

Table 3. *Cont.*

Cohort and Study Details	Flavonol Intake	Aim	Bioavailability Data	Outcome	Reference
229 healthy subjects (mean age 31.05 ± 8.9 years) Duration: 3 months Randomized double-blind, placebo controlled study	Participants consumed 16.7 mg/day of sea buckthorn extract or placebo, added to 28 g of puree. The daily dose of sea buckthorn extract contained: - 5.8 ± 0.7 mg isorhamnetin 3-O-glucoside-7-O-rhamnoside; - 1.5 ± 0.9 mg quercetin 3-Orutinoside; - 1.6 ± 0.4 mg quercetin 3-O-glucoside; - 5.1 ± 0.8 mg isorhamnetin 3-O-rutinoside; - 2.4 ± 0.4 mg isorhamnetin 3-O-glucoside; - 0.3 ± 0.4 mg kaempferol 3-O-rutinoside.	To study the effect of flavonoid-rich sea buckthorn berry on circulating lipid markers associated with CVD risk (total, HDL and LDL cholesterol, triacylglycerols) and CRP.	The consumption of sea buckthorn extract significantly modified the plasma concentration in treated group: ↑ quercetin (3.0 ng mL^{-1}, $p = 0.03$); ↑ isorhamnetin (3.9 ng mL^{-1}, $p < 0.01$).	Sea buckthorn extract did not affect serum concentration of any CVD risk factors considered.	[43]
15 healthy volunteers (6 M; 9 F) (mean age 60.8 ± 9.3 years) Duration: 1 week Randomized, controlled, crossover study	Each subject received 5 doses of quercetin-3-O-glucoside: - 0 mg; - 50 mg; - 100 mg; - 200 mg; - 400 mg. Each compound (control or treatment) was provided once in the morning in a cup of coffee.	To determine whether endothelial function, BP and NO were affected in a dose-dependent mode of administration of quercetin-3-O-glucoside.	After the intake of increasing doses of quercetin-3-O-glucoside, was observed: ↑ quercetin dose-dependent plasma concentrations ($R^2 = 0.52$, $p < 0.001$), ↑ isorhamnetin dose-dependent plasma concentrations ($R^2 = 0.12$, $p = 0.005$). Baseline: - free quercetin 1.90 ± 1.1 mM; - isorhamnetin 0.99 ± 0.06 mM.	After any intervention, no improvements were observed in: - endothelial function, - BP; - NO production.	[47]
6 healthy subjects (4 M; 2 F) (mean age 34 ± 7 years) Duration: 1 day Randomized placebo-controlled crossover study	Participants were randomly treated with the following: - 150 mg Q-4-G in 5% ethanol; - 300 mg Q-4-G in 5% ethanol; - 5% (*v*/*v*) ethanol control drink.	To investigate the effect of the dietary ingestion of quercetin on platelet function (platelet aggregation and platelet collagen-stimulated tyrosine phosphorylation).	Plasma concentrations peaked 30 min after ingestion. Group 150 mg: - Quercetin 4.66 ± 0.77 µM; - Isorhamnetin 0.16 ± 0.05 µM; - Tamarixetin 0.24 ± 0.07 µM; - Total flavonoid 5.07 ± 0.90 µM. Group 300 mg: - Quercetin 9.72 ± 1.38 µM; - Isorhamnetin 0.44 ± 0.07 µM; - Tamarixetin 0.54 ± 0.09 µM (after 45 min); - Total flavonoid 10.66 ± 1.55 µM. These results indicating dose-dependent bioavailability of flavonoid.	After 30 and 120 min since intake of both doses of Q-4-G were inhibited: - platelet aggregation ($p = 0.001$); - collagen-stimulated tyrosine phosphorylation of TPP ($p = 0.001$).	[48]

Table 3. *Cont.*

Cohort and Study Details	Flavonol Intake	Aim	Bioavailability Data	Outcome	Reference
12 healthy men (mean age of 43.2 ± 4.3 years) Duration: acute consumption Randomized, placebo-controlled, crossover trial	Each participant received, in random order, 4 treatments: - 300 mL water (control); - 0.67 mg/mL quercetin; - 0.67 mg/mL epicatechin; - 0.67 mg/mL EGCG.	To evaluate the effects of quercetin and epicatechin on the endothelial function (measuring endothelin-1 and NO production) and oxidative stress (measuring urinary F2-isoprostanes).	Acute treatment with quercetin and epicatechin significantly increased ($p < 0.001$) the total circulating concentration of each flavonoid (from 0.84 ± 0.39 µmol L^{-1} to 3.54 ± 1.57 µmol L^{-1} for quercetin and from and 0.70 ± 0.34 µmol L^{-1} to 3.57 ± 1.21 µmol L^{-1} for epicatechin). In urine, concentrations of total quercetin increased from 0.61 ± 0.15 to 2.51 ± 0.65 µmol $mmol^{-1}$ creatinine and total epicatechin from 0.50 ± 0.28 to 2.62 ± 0.98 µmol $mmol^{-1}$ creatinine ($p < 0.001$). Plasma concentrations of EGCG increased from 0.06 ± 0.01 to 0.10 ± 0.01 µmol L^{-1} ($p < 0.05$). EGCG was not detected in urine.	EGCG did not affect NO production. Quercetin and epicatechin significantly reduced plasma endothelin-1 concentration ($p < 0.05$), but only quercetin significantly decreased the urinary endothelin-1 concentration. None of the 3 treatments significantly decreased plasma or urinary F2-isoprostane concentrations.	[49]
15 healthy subjects (9 M; 6 F) (mean age 25.8 ± 5.2 years) Duration: 3 weeks Double blind, randomized, placebo-controlled trial.	Subjects received a capsule containing the following: - Placebo; - 200 mg of quercetin; - 400 mg of quercetin.	To evaluate whether the deconjugation of quercetin-3-O-glucuronide (Q3GA) may improve vasodilator effects of quercetin.	At 2 h post ingestion, plasma levels were as follows: - 200 mg quercetin group: 0.35 µM Q3GA, 0.043 µM quercetin aglycone, 0.008 µM isorhamnetin aglycone, - 400 mg quercetin group: 0.95 µM Q3GA, 0.031 µM quercetin aglycone, 0.035 µM isorhamnetin aglycone. Glucuronides of isorhamnetin were not detected.	After ingestion (2 or 5 h) of both doses, were not changes in systolic and diastolic blood pressure. A time-dependent increase in brachial artery diameter was detected after 400 mg quercetin intake, correlated with the levels of Q3GA mediated by glucuronidase activity.	[50]

CRP, C reactive protein; CVD, cardiovascular disease; BP, blood pressure; FMD, flow-mediated dilatation; FPG, fasting plasma glucose; sBP, systolic blood pressure; HF, high-flavonoid; LF, low flavonoid; LOD, Limit of Detection; EGCG, Epigallocatechin gallate; Q-4-G, quercetin-4-O-β-glucoside; TPP, total platelet proteins.

Despite the negative results, plasma flavonols (quercetin, kaempferol and isorhamnetin glucuronides, and sulfates) significantly increased after the treatment, with respect to placebo ($p < 0.005$). This means that the initial dose taken correlates closely with the biological effects but is not always explained by the bioavailability of flavonols and the duration of intake. In fact, although the flavonol plasma levels were always measurable, they were not sufficient to affect specific biomarkers. Quercetin, especially in its glucosidic form, is generally efficiently absorbed and bioavailable. In addition, quercetin metabolite elimination is quite low ($t_{1/2}$ 11–28 h), leading to their accumulation in plasma with repeated intakes [20]. Positive results were obtained on FMD and platelet aggregation.

Bondonno et al. (2016) observed no improvement in blood pressure or FMD in healthy subjects, when increasing amounts of quercetin-3-O-glucoside (50–400 mg/day) were provided [47], but only when quercetin was enzymatically modified to obtain a more bioavailable α-oligoglucosylated quercetin derivative, isoquercitrin. Supplementation with 4.89 mg of this compound significantly increased FMD response by 1.8% compared with the placebo ($p = 0.025$), but with different mechanisms not involving NO production increase. In contrast, cognitive function was not affected. The different form of quercetin used makes it difficult to compare it with other studies; however, the maximum plasma concentration reached 144 ± 12.3 nM, which is higher than the values measured in other studies where quercetin was administered [48,49].

Perez et al. (2014) postulated that the vasodilator effects can be mediated by quercetin-3-O-glucuronide (Q3GA) deconjugation mediated by plasmatic glucuronidase [50]. This is because a plasmatic dose-dependent increase in this metabolite was detected after 200 and 400 mg supplementation with quercetin, which was not detected with other metabolites (quercetin aglycone, isorhamnetin aglycone, and their glucuronide forms). The enzyme glucuronidase is present in lysosomes involved in the glycosaminoglycans cleavage. An extensive inter-individual variability in the activity of glucuronidase has also been described, which may be attributed by variations in its gene sequence or expression. This enzyme hydrolyzes glucuronidated metabolites at the vascular level, producing the parent aglycone, which, due to its elevated liposolubility, accumulates in tissues and performing its biological activity. The effect on arterial diameter was not associated with either the early changes flavonoid plasmatic levels or the glucuronidase activity. Nevertheless, these effects were associated with a combination of both factors. Quercetin provided by food (high-quercetin onion soup) or supplements (providing 138 and 150 mg quercetin, respectively) was shown to be effective in inhibiting platelet aggregation mediated by collagen. The inhibition correlated highly with the AUC of the quercetin metabolites, isorhamnetin and tamarixetin (4-O-methyl-quercetin) [40,48].

3.4. Phenolic Acids

Phenolic acids are a class of secondary metabolites, highly distributed among plants. According to their chemical structure, phenolic acids can be divided in benzoic and cinnamic acids. The main benzoic groups are gallic, protocatechuic, and *p*-hydroxybenzoic acids, mainly as conjugates. The highest concentration (fresh weight) of benzoic acids has been calculated in Apiaceae species (spices and herbs): anise 730–1080 mg kg^{-1}, cumin up to 42 mg kg^{-1}, fennel up to 106 mg kg^{-1}, and parsley up to 30 mg kg^{-1} [51,52]. Cinnamic acids are widely distributed in plants, as esters or amides. The most representative are caffeic, chlorogenic, and ferulic acids. High concentrations of cinnamic acids are coffee, tea, wine, cocoa, fruits, vegetables, and cereals. Some of the most important sources of caffeic acid are wild blueberry (1470 mg kg^{-1}), coffee (870 mg kg^{-1}), carrots (260 mg kg^{-1}), plum (234 mg kg^{-1}), and eggplant (210 mg kg^{-1}).

One of the most important derivatives of caffeic acid is caftaric acid, a representative polyphenol in wine (6–73 mg L^{-1} in white wine, 46–141 mg L^{-1} in red wine), while chlorogenic acid is present in

considerable levels in coffee (depending on the climatic and processing conditions, and procedures for coffee preparation) [53,54]. The chlorogenic acid content in roasted coffee beans varies depending on the roasting extent, in the range of 2.3–80 g/kg (dried weight) and 890–8130 mg/L in espresso coffee [53]. The intake of chlorogenic acid can be very high; it has been estimated to be up to 0.8 g per day among coffee drinkers [20].

Cereals are the most important source of ferulic acid, derivative of cinnamic acid derivative (for which the intake ranges from about 0.092 to 0.32 g) [49]. Table 4 shows the studies correlating phenolic acid intake, their bioavailability, and different health effects, mainly focused on blood pressure, vasodilation, antioxidant activity, and inflammation. Six of ten studies included in this review investigated the effects of chlorogenic acid and its metabolites in coffee or in beverages prepared in order to mimic coffee intake; one study used purified caffeoylquinic acid (5-CQA); one study included whole grain; and two studies included a blueberry drink. Phenolic acids were administered to healthy subjects in the range of 138.7 and 900 mg/day and were tested both in acute and chronic consumption (maximum eight weeks). Generally speaking, vascular function was positively affected by chlorogenic acid (CGA) provided by decaffeinated coffee intake (50 mL) or purified caffeoylquinic acid (5-CQA), confirming previous studies showing that phenolic compounds, other than caffeine, can contribute to vasoactive efficacy [55,56]. Observational studies indicate that moderate coffee intake (4 cups), containing from 105 to 500 mg of CGA, is associated with a lower CVD risk [57].

Potential mechanisms by which CGA and its main plasma metabolites (5-cholorgenic acid, ferulic-4′-O-sulfate, and isoferulic-3′-O-glucuronide) mediate the vascular effects include the inhibition of NAPDH oxidase, leading to a reduction in superoxide production and, as a consequence, to an increase in endothelium NO bioavailability [58]. Conflicting positions have been taken regarding the possible agonistic or antagonist effects of caffeine on vascular function. Agudelo-Ochoa et al. (2016), postulated that caffeine could be responsible for the negative effects observed after 400 mL coffee intake (caffeine content < 300 mg), since it can interfere with the mechanism of action of chlorogenic acid, thus decreasing NO production [59]. In contrast, Boon et al. (2017) noticed a vasodilator effect only in subjects consuming caffeinated coffee (270 mg caffeine) but not decaffeinated coffee, although the CGA levels were comparable (300 and 287 mg CGA, respectively) [60]. In plasma, an increase in CGA metabolites (5-CGA) was always measured, even when no significant effects on vascular function were observed, suggesting that synergistic effects on different polyphenol compounds can occur. Rodriguez-Mateos et al. (2013) also observed an amelioration of endothelial function after the acute intake of blueberry drinks containing different levels of polyphenols, from 766 to 1791 mg [61]. Phenolic acid metabolites (caffeic acid, ferulic acid, iso-ferulic acid, vanillic acid, benzoic acid, and 2-hydroxybenzoic acid) were the only ones measured in plasma, while no flavonols or anthocyanins were detected, thus suggesting that these compounds were not responsible for the positive effects noticed. The intake of phenolic acids with whole grains (138 mg/70 g) for eight weeks was associated with a reduced inflammatory status in overweight subjects by Vitaglione et al. (2015), compared with equal amounts of refined wheat (2.6 mg ferulic acid) [62].

Table 4. Phenolic acids' beneficial effects and bioavailability in human subjects.

Cohort and Study Details	Phenolic Acids Intake	Aim	Bioavailability Data	Outcome	Reference
1° study: 15 healthy male subjects (mean age: 26.3 ± 1.6 years) 2° study: 24 healthy male subjects (mean age: 23.8 ± 1.4 years) Duration: acute consumption Randomized controlled crossover studies	1° study: Subjects consumed 50 mL coffee containing high (310 mg) or low (89 mg) chlorogenic acid levels. Control intervention contained 0 mg chlorogenic acid. 2° study: Subjects consumed (a) 0.45 g purified 5-caffeoylquinic acid (5-CQA) + 1 g maltodextrin (MDX); (b) 0.90 g purified 5-CQA + 1 g MDX; 1 g MDX (negative control) and 0.20 g purified epicatechin + 1 g MDX (positive control). Each preparation was solubilized in 200 mL of hot water.	To evaluate the vascular function (% flow-mediated dilation, FMD) after the consumption of coffee rich in chlorogenic acid.	1° study: After 1 h of treatment was observed, a significant positive correlation between total plasma chlorogenic acids metabolites (CgAM) and % FMD: 3-caffeoylquinic acid (3CQA) 4-caffeoylquinic acid (4CQA), 5-feruloylquinic acid (5FQA) and caffeic-3′-O-sulfate (CA3S) ($p < 0.005$). After 5 h, where a second peak of % FMD was observed, the correlation was significant for ferulic-4′-O-sulfate, isoferulic-3′-O-glucuronide, caffeic-4′-O-sulfate, m-coumaric acid-3′-O-sulfate and 4-methoxycinnamic acid ($p < 0.005$). 2° study: Total plasma CgAM post intervention of 0.45 g of chlorogenic acids were lower than following intake of the 0.90 g dose, but both significantly ↑ ($p = 0.032$ and $p = 0.006$, respectively).	1° study: Both low and high chlorogenic enriched coffee improved vascular function 2° study: Only after the 0.45 g dose of purified 5-CQA, %FMD ↑ in comparison to the water control (6.02 ± 0.28 to 6.77 ± 0.42, $p = 0.06$), whereas intake of the control (5.90 ± 0.33% to 5.86 ± 0.30%) or the higher dose of 5-CQA (0.900 g) had no effect on %FMD (6.17 ± 0.31% to 6.46 ± 0.35%).	[55]
23 healthy subjects (4 M; 19 F) (mean age: 52.3 ± 10.6 years) Duration: acute consumption Randomized, double-blind, placebo-controlled, crossover trial	The treatments included: - water (used as control); - 0.40 g of chlorogenic acid (3-O-caffeoylquinic acid) solubilized in 0.20 L of water (corresponding to 2 cups of coffee).	To investigate the acute effects of chlorogenic acid on different parameters: - Nitric oxide level; - Endothelial function; - Blood pressure.	Chlorogenic acid concentration in plasma was significantly higher 150 min after the consumption of 0.40 g of CGA as compared to control group ($p < 0.001$). No significant differences were observed in term of chlorogenic acid metabolites (isoferulic acid, ferulic acid, phloretic acid, caffeic acid, and hydrocaffeic acid) between the two treatments.	The mean systolic blood pressure (−2.41 mmHg, $p = 0.05$) and diastolic blood pressure (−1.53 mmHg, $p = 0.04$) were significantly ↓ in the chlorogenic acid group compared with control group. NO levels ($p > 0.10$) and endothelial function ($p = 0.60$) were not significantly affected.	[56]

Table 4. *Cont.*

Cohort and Study Details	Phenolic Acids Intake	Aim	Bioavailability Data	Outcome	Reference
75 healthy subjects (38 M; 37 F) (mean age: 38.5 ± 9 years) Duration: 8 weeks Randomized, placebo-controlled trial	Group A: consumption 400 mL coffee/day containing high levels chlorogenic acids (HCCGA) (780 mg/400 mL); group B: (400 mL coffee/day containing medium levels chlorogenic acid (MCCGA) (420 mg/400 mL); group C: placebo. Chlorogenic acids were composed by: 5-O-caffeoylquinic; 3-O-caffeoylquinic; 4-O-caffeoylquinic; caffeic; ferulic; 3,4-di-O-caffeoyl-quinic; 3,5-di-O-caffeoylquinic; and 4,5-di-O-caffeoylquinic.	To evaluate the effects of chlorogenic acids on the following: - The antioxidant capacity of plasma; - The lipid profile in serum; - The vascular function (flow-mediated dilation-FMD, nitric oxide levels, blood pressure).	The concentration of ferulic and caffeic acid was ↑ in the groups that consumed coffee-drinking and were significantly higher 1 h after the consumption in the MCCGA group than the HCCGA group (caffeic acid: 50.5 ± 6.9 nM vs 20.3 ± 3.3 nM; ferulic acid: 201 ± 18.7 nM vs. 137 ± 6.1) ($p < 0.001$). In both groups the ferulic and caffeic acids decreased after 8 weeks.	Plasma antioxidant capacity significantly increased only in the group consuming medium levels of chlorogenic acid (6%) and group consuming high levels chlorogenic acids (5%) ($p < 0.05$). No effects on lipid profile and vascular function were measured.	[59]
16 healthy subjects (10 F, 6 M) (mean age: 59.9 ± 8.2 years) Duration: acute consumption Double-blind, randomized, placebo-controlled crossover study	Subjects received: (1) 0 g purified 5-chlorogenic acid (p5-CgA) (control group); (2) 0.45 g p5-CgA; (3) 0.90 g p5-CgA; and (4) 0.20 g purified (−)-epicatechin (positive control). Each treatment was prepared in 200 mL of warm water and consumed within 10 min. A minimum one-week washouts between visits was required.	To evaluate the acute effect of two doses of (5-CgA) (0.45 and 0.90 g) on vascular function (FMD) and blood pressure.	After 1 h and 4 h from the consumption of 0.90 g 5-CgA, total CgA metabolites, reached 1.5 µM and 1.25 µM, respectively. After 0.45 g, metabolites reached 0.75 µM and 1 µM after 1 and 4 h, respectively. In both cases, the most representative CgA metabolite was 5-caffeoylquinic acid (5-CqA).	None of the doses of 5-CgA used significantly affected FMD response.	[63]
12 healthy subjects (5 F, 7 M) (mean age: 59.4 ± 6.4 years) Duration: acute consumption Randomized, placebo-controlled, crossover trial	Subjects consumed: (1) 18 g of ground caffeinated coffee + 0.3 g CgA in 0.20 L of hot water; (2) 18 g of decaffeinated coffee + 0.287 g CgA in 0.20 L of hot water; (3) 0.20 L of hot water (control). For each group the beverages were consumed twice. The second beverage was consumed after 2 h with a 75 g glucose load.	The aim of the study was to evaluate the effect of coffee on different outcomes: (1) vascular function (FMD), (2) blood pressure, (3) glucose concentration in blood.	The mean values concentration for 5-CgA were: - caffeinated group: 1.89 ± 0.56 nM; - water group: 1.21 ± 0.22 nM; - decaffeinated group: 1.30 ± 0.29 nM. No significant difference in 5-CgA concentration between the three groups were observed.	The FMD response was significantly ↑ in the caffeinated coffee group compared to the other groups (decaffeinated coffee and water) ($p < 0.001$). No significant difference in the FMD response between decaffeinated coffee and water groups was observed. No differences were observed in term of blood glucose concentrations and blood pressure between the three groups considered.	[60]

Table 4. *Cont.*

Cohort and Study Details	Phenolic Acids Intake	Aim	Bioavailability Data	Outcome	Reference
68 healthy overweight/obese subjects (23 M; 45 F) (mean age: 38.5 ± 2 years) with sedentary lifestyle and reduced intake of fruit and vegetables Duration: 8 weeks Randomized placebo-controlled, randomized trial	Whole grain (WG) group consumed 70 g wheat/day containing 96.7 mg ferulic acid, 26.5 mg sinapic acid, 9.4 mg coumaric acid, 1.9 mg gallic acid, 1.8 mg syringic acid, 1.6 mg vanillic acid, 0.5 mg salicylic acid, 0.3 mg caffeic acid); control group (CTR) consumed 60 g refined wheat (RW) products/day containing 2.6 mg ferulic acid.	To investigate the role of whole grain (WG) consumption on plasma markers of metabolic disease and inflammation (tumor necrosis factor-α (TNF-α) interleukin-10 (IL)-10), plasminogen activator inhibitor 1.	After 8 weeks, WG consumption was associated with a 4-fold ↑ in serum dihydroferulic acid (DHFA) and a 2-fold ↑ in fecal ferulic acid (FA) compared with RW group. Similarly, after 8 weeks, urinary FA was 2-fold ↑ the baseline concentration only in WG group.	In the WG group was observed a ↓ in TNF-α after 8 weeks and ↑ IL-10 only after 4 weeks, compared with RW group ($p = 0.04$). No significant differences in plasma metabolic disease markers were observed.	[62]
47 habitual coffee drinkers at risk for type-2 diabetes (11 M; 36 F) (mean age 54.0 ± 9.0 years) Duration: 3 months Crossover clinical trial	The coffee consumption during the trial was set as follow: - First month: subjects avoided to drink coffee; - Second month: subjects consumed 4 cups (150 mL/cup) of filtered coffee/day; - Third month: participants consumed 8 cups of filtered coffee/day.	To evaluate the role of daily coffee consumption on the following: - The modulation of different biomarkers of inflammation (interleukin-18 (IL-18), 8-isoprostane, and adiponectin); - Oxidative stress and glucose; -Lipid metabolism.	Positive correlations between the ↑ in serum concentrations of coffee phenolic acid metabolites (cPAM) and changes in adiponectin concentrations were detected after 8 cups coffee/day consumption vs. baseline (0 cups coffee/day). Significant correlations were observed for isoferulic acid ($r = 0.328$, $p = 0.025$) and dihydroisoferulic acid ($r = 0.323$, $p = 0.027$). Negative or no correlation was observed for the other cPAM.	Significant differences were observed for serum concentrations of IL-18, 8-isoprostane, only after 8 cups coffee/day consumption compared with baseline (0 cups coffee/day). Serum concentrations of total cholesterol, HDL cholesterol, and apolipoprotein A-I ↑ significantly (+12%, 7%, and 4%, respectively). The ratios of LDL to HDL cholesterol and of apolipoprotein B to apolipoprotein A-I significantly ↓ (−8% and 9%, respectively) after the consumption of 8 cups coffee/day compared with 0 cups coffee/day. No variations were observed for glucose metabolism markers.	[64]

Table 4. *Cont.*

Cohort and Study Details	Phenolic Acids Intake	Aim	Bioavailability Data	Outcome	Reference
20 healthy subjects (6 M; 14 F) (mean age: 35.7 ± 9.0 years) Duration: acute consumption Randomized placebo-controlled trial	0.4 L of Arabica coffee containing 0.42 g CgAs (6 g/100 mL: provided 105 ± 4.1 mg of CgAs) were given to the intervention group The sum of the following acids contributed to the total CgAs and phenolic acids content: 5-O-caffeoylquinic; 3-O-caffeoylquinic; 4-O-caffeoylquinic; caffeic; ferulic; 3,4-di-O-caffeoylquinic; 3,5-di-O-caffeoylquinic; and 4,5-di-O-caffeoylquinic. No coffee or placebo were consumed by the control group.	The caffeic acid (CA) and ferulic acid (FA) were quantify and different methods were applied to evaluate the antioxidant capacity of plasma.	The concentrations of FA and CA, measured 1 h after the coffee consumption, were: 202.38 ± 12.87 nM and 49.76 ± 6.44 nM, respectively. Both acids (CA and FA) were not detected at baseline. In the control group, at 1 h, the same trend was observed.	The antioxidant capacity of plasma was measured with ferric reducing antioxidant power assay (FRAP) and oxygen radical absorbance capacity assay (ORAC). Compared to the baseline, the antioxidant capacity, measured with both methods, ↑ significantly (+6.67%; $p < 0.001$ for FRAP and +7.16%; $p < 0.05$ for ORAC). A correlation was observed between the ↑ of antioxidant capacity and the plasma concentration of FA and CA.	[65]
21 healthy men (mean age: 27 ± 1.3) Duration: acute consumption Randomized, controlled, double-blind, crossover human- intervention trials	1° study: participants consumed: - A blueberry drink containing: 0.766, 1.278, and 1.791 g total blueberry polyphenols (corresponding to 0.24, 0.40, and 0.56 kg fresh blueberries, respectively); - Control drink (macronutrient and micronutrient drink). 2° study: participants consumed the following: - A blueberry drink containing 0.319, 0.637, 0.766, 1.278 and 1.791 g total blueberry polyphenols (corresponding to 0.10, 0.20, 0.24, 0.40, and 0.56 kg fresh blueberries, respectively); - Control drink (macronutrient and micronutrient drink).	The endothelial function (FMD) was monitored and the time-dependent (1° study) and intake-dependent (2° study) changes were investigated.	A correlation between the FMD ↑ and the plasma concentration ↑ of different phenolic acid metabolites (ferulic acid, isoferulic acid, vanillic acid, 2-hydroxybenzoic acid, benzoic acid, and caffeic acid—sum of conjugated and nonconjugated compounds) were observed 2 and 6 h after consumption. The phenolic acid metabolites were significantly ↑ at 1–2 h after blueberry polyphenol consumption ($p < 0.001$). The plasma total concentration of metabolites was about 400 nmol/L (coinciding with the highest FMD value at 1 h). The metabolites of flavanol or anthocyanin were not detected in plasma at any time after the intake of blueberry drink.	The consumption of 0.10–0.24 kg blueberry (corresponding to 0.319, 0.639 and 0.766 g total polyphenols) positively affected vascular function.	[61]

CgA, Chlorogenic Acid; HCCGA, high levels chlorogenic acids; MCCGA, medium levels chlorogenic acid; WG, whole grain.

Considering the bioavailability data, this effect was mainly associated with ferulic acid, whose concentrations were two-fold higher in the feces of subjects consuming whole grains. Interestingly, this result was explained by the fact that the release of FA in the colon could be due to wheat bran polysaccharide fermentation and mediated by bacterial enzymes xylanase and ferulic acid esterase. These enzymes are mainly synthetized by Lactobacilli (Firmicutes), Bifidobacteria (Actinobacteria), Bacteroides, and Prevotella (Bacteroidetes) when arabinoxylans with esterified ferulic acids are introduced [66,67]. Since overweight or obese individuals show reduced amounts of Bacteroidetes and Bifidobacteriales, Firmicutes are considered the main responsible for the fermentation of whole-grain polysaccharides and ferulic acid liberation [62,66].

3.5. Stilbenes, Isoflavones, and Flavanones

Only a few studies were found correlating the effects of stilbenes (resveratrol), isoflavones, and flavanones with their bioavailability (Table 5). Resveratrol is a phytoalexin that is widely distributed in the plant kingdom. It is found in more than 70 species, but grapes and wine are the most important sources. The mean levels of total resveratrol in red wine is 7 mg L^{-1}; rose wine has a total of 2 mg L^{-1}, and white wine has and 0.5 mg L^{-1} [60]. Resveratrol supplementation (250–500 mg/day for 7 and 28 days, respectively) was investigated in healthy subjects as regards cognitive function and cerebral ematic circulation. Despite total resveratrol metabolites (resveratrol 4′glucuronide, 3′ glucuronide, and sulfate) being ten-fold higher in the treatment group, supplementation failed to improve cognitive function but increased cerebral flow and reduced fatigue levels [68,69]. These effects could also be mediated by unmetabolized resveratrol, since the literature data indicate that it can be found in plasma bound to albumin or LDL, and that it elicits its biological function after interaction with cells that have receptors for albumin and LDL [70]. In brain, resveratrol contributed to vasorelaxation, oxygenation, and sirtuin (SIRT)-mediated increases in mitochondrial gene expression in brain [68].

Table 5. Stilbenes', isoflavones', and flavanones' beneficial effects and bioavailability in human subjects.

Cohort and Study Details	Phenols Intake	Aim	Bioavailability Data	Outcome	Reference
• **Stilbenes**					
60 subjects (9 M; 51 F) (mean age: 20.52 years) Duration: 28 days Randomized, double-blind, placebo-controlled, parallel-groups study	500 mg/day of pure trans-resveratrol (also containing 10 mg of piperine/capsule) or a placebo.	To evaluate the effect of resveratrol on cognitive performance (measured as serial subtractions, rapid visual information processing, 3 Back test), mood, sleep quality and cerebral blood flow (CBF).	Resveratrol 3-O-sulfate was the predominant metabolite in all volunteers, contributing 73–77% of total metabolites, followed by resveratrol 4′ glucuronide and 3′ glucuronide. Total resveratrol metabolites increased in plasma from 3 to 13 μM 110 min after administration.	Although stilbene metabolite levels increase in plasma, supplementing with 500 mg of resveratrol for 28 days did not improve cognitive function.	[68]
22 healthy subjects (4 M; 20 F) (mean age: 20.17 years) Duration: 7 days Randomized, double-blind, placebo-controlled, crossover study	Subjects received the following treatments: - inert placebo; - 250 mg trans-resveratrol; - 500 mg trans-resveratrol;	To evaluate the effects of oral resveratrol on cognitive performance and CBF. In a separate group ($n = 9$) was investigated plasma levels of resveratrol and its conjugates after the intake of the same treatments.	Resveratrol sulfate and glucuronide were the main metabolites and reached a peak plasma concentration at 90 min after both 250 and 500 mg supplementation. As regards unmetabolized resveratrol, 90 min after both supplementations, reached low concentrations, peaking at 5.65 and 14.4 ng mL^{-1}, respectively.	CBF increased in a dose-dependent fashion of resveratrol intake during task performance. No changes in cognitive function were registered.	[69]
• **Isoflavones**					
10 overweight or obese men (mean age: 56.2 ± 6.18 years) Duration: acute consumption Randomized, double-blind, placebo-controlled, crossover study	Subjects consumed a high-fat, high-fructose breakfast with 4 dietary supplementations: - Placebo: fish oil placebo and isoflavone placebo; - FO: fish oil and isoflavone placebo; - ISO: fish oil placebo and isoflavones; - FO + ISO: fish oil and isoflavones. The soy isoflavone supplements provided 150 mg glycoside isoflavones (eq. to 96 mg aglycone form) in proportions of 1.05/1.0/0.29 for genistein/daidzein/glycitein. Fish oil supplement (1 g of refined fish oil concentrate) providing 0.4 g EPA and 0.2 g DHA.	To evaluate the effect of acute supplementation with fish oil (n-3), PUFA, soy isoflavones, and their combination on postprandial serum triglycerides (TG) and oxidative biomarkers in a proatherogenic high-fat, high-fructose meal.	At 4 h, postprandially serum concentration was as follows: - Genistein ISO: 1.027 ± 0.122 μmol L^{-1}, FO + ISO: 1.185 ± 0.079 μmol L^{-1}; - Daidzein ISO: 0.838 ± 0.096 μmol L^{-1}, FO + ISO: 1.017 ± 0.046 μmol L^{-1}.	The high-fat, high-fructose meal significantly increased serum total FA and TG without affecting oxidative stress biomarkers. Serum TG and oxidative stress biomarkers did not differ between treatments. The FO and ISO were bioavailable but did not reduce the postprandial rise in serum TG. Neither the study meal nor the FO or ISO induced significant changes in oxidative stress.	[71]

Table 5. *Cont.*

Cohort and Study Details	Phenols Intake	Aim	Bioavailability Data	Outcome	Reference
78 postmenopausal osteopenic women (mean age: 61.85 ± 1.03 years) Duration: 12 months Double-blind, parallel design, placebo-controlled trial	Participants received supplementation: 1.2 g/day calcium, 0.55 g/day magnesium, 0.025 g/day calcitriol, and a red clover extract (0.06 g/day isoflavone aglycones and probiotics) or a placebo.	To determine the beneficial effects of a bioavailable isoflavone and probiotic treatment in postmenopausal osteopenia.	After 12 month, isoflavone concentration in the treated group was 3.933 μg mL^{-1} (median), significantly higher from baseline ($p = 0.0094$) (compared with the control group, where median values were 2.323 μg mL^{-1}).	Treatments with red clover extract: ↓ BMD loss, ↓ plasma concentrations of collagen type 1 crosslinked C-telopeptide ($p < 0.05$).	[72]
237 women (mean age: 53 ± 3 years) Duration: 12 months Randomized, double-blind, placebo-controlled, parallel, multicenter trial (including the Netherlands, Italy and France)	Subjects, during their habitual diet and lifestyle, consumed 110 mg/day isoflavone aglycones or control.	To evaluate whether bone metabolism and mineral density, and hormonal conditions were affected by chronic consumption of isoflavone-enriched foods.	↑ isoflavones plasma levels in treated group. Both genistein and daidzein were higher in the Netherlands (1522 ± 1136.2 nmol L^{-1} and 338.2 ± 261.3 nmol L^{-1}, respectively) than in France (533.4 ± 607.4 nmol L^{-1} and 92.9 ± 145.8 nmol L^{-1}, respectively) and Italy (541.5 ± 557.6 nmol L^{-1} and 133.4 ± 188.3 nmol L^{-1}, respectively).	Bone mineral density or biomarkers of bone were not affected by isoflavone-enriched products chronic intake. Hormone concentrations did not differ between the two groups.	[73]
• **Flavanones**					
16 men at moderate CVD risk (mean age: 60.6 ± 1.4 years) Duration: acute consumption Randomized, placebo-controlled crossover trial	Participants received 767 mL orange juice or a hesperidin supplement (both providing 320 mg hesperidin and 439 mg vitamin C) or control.	To evaluate the effects of orange juice or a hesperidin supplement on plasma concentrations of flavanone metabolites and their effects on cardiovascular risk biomarkers (blood pressure, endothelial function, central arterial stiffness, cardiac autonomic function, platelet activation, and NADPH oxidase gene expression).	After 5 h from the orange juice intake, significantly increased plasma concentrations of 8 flavanones (1.75 ± 0.35 mmol L^{-1}, $p < 0.0001$), and 15 other phenolic metabolites (13.27 ± 2.22 mmol L^{-1}, $p < 0.0001$) were significantly increased. In particular, 47% hesperidin-glucuronide, 15% naringenin-7-O-glucuronide, 14% a second hesperidin-glucuronide, 54% hippuric acid, 15% dihydroferulic acid, 8% dihydroferulic acid–glucuronide, 7% 4-hydroxyphenylacetic acid, and 5% vanillic acid increase were detected.	Effects on CVD risk factor were not observed.	[74]

BMD, bone mineral density; CBF, cerebral blood flow; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; NADPH, nicotinamide adenine dinucleotide phosphate.

The only source of isoflavones are products derived from soybeans. Depending on the kind of soy preparation, isoflavones can be present as aglycones or glycosides. One study evaluated the impact of isoflavones on triglycerides and oxidative biomarkers [71]. Only two studies investigated the effects of isoflavones (supplemented for 12 months at doses of 60 and 96 mg isoflavone aglycones/day, respectively) on bone density in post-menopausal women with contradictory results. In the study by Lambert et al. (2017), the positive effects were explained by the use of lactic acid probiotic bacteria in the treatment group in association with isoflavones, which mediated equol production [72]. Equol is a derivative of daidzein produced by anaerobic bacteria with great estrogenic potential. Since none of the subjects was able to produce equol at the beginning of the study and, after six months of treatment, 55% of individuals in the Red Clover Extract (RCE) group produced equol, it is plausible that that the probiotics positively affected the participant intestinal bacterial pattern, promoting more positive conditions for equol production. This hypothesis seems to be confirmed by the fact that high plasma levels were found in both studies; however, in the study by Brink et al. (2008), bone density was not affected by isoflavones [73]. However, a higher number of studies is necessary to confirm these results.

Only one study investigated the effect of flavanones, in particular hesperidin on vascular function, providing 320 mg with 767 mL orange juice/day as acute consumption. Despite the detection of several hesperidin metabolites in plasma (hesperidin-glucuronide, naringenin-7-O-glucuronide, dihydroferulic acid, dihydroferulic acid–glucuronide, hippuric acid, and vanillic acid 4-hydroxyphenylacetic acid) after 5 h, no significant effects were measured. Despite previous studies showing that plasma hesperidin metabolites were correlated with health effects on the endothelium after flavanone acute consumption [75, 76], further investigations are necessary to evaluate and support the biological effects of these polyphenols.

4. Conclusions

The data reported in this review highlight that, despite the very large number of studies investigating the health effects of polyphenols in humans, only a few have considered their bioavailability in order to partially support the associated bio-efficacy. The bioavailability of polyphenols varies among the different classes and ranks as follows: phenolic acids > isoflavones > flavonols > catechins > flavanones, proanthocyanidins > anthocyanins, confirming data from previous pharmacokinetic studies [20]. Apart from favanols and flavonols, the amounts of polyphenols used in studies were considered to be too low to reach significant plasma concentrations that would provide beneficial effects. However, the amounts were chosen on the basis of the mean consumption in the daily diet of the populations included in the studies. Another point to be considered is that healthy subjects were mainly included in clinical trials. On the one hand, it makes it more difficult to measure significant changes in biomarkers generally associated with pathological conditions; on the other hand, it suggests a potential role of polyphenols provided with the daily diet or supplementation in maintaining the health status. In fact, positive variations of physiological parameters generated by polyphenol intake could help in improving or modulating specific functions and reducing some risk factors for chronic diseases. Cardiovascular function was the main health area investigated. Vasodilator effects were found for phenolic acids (mainly chlorogenic acid and ferulic acid) and flavanols (in particular catechins and proanthocyanidins), which were partially explained by their bioavailability.

As regards anthocyanins, their plasmatic levels were too low to affect the biomarkers considered; however, cyanidin and peonidin were the most available. Future research should focus on confirming and integrating the data discussed in this review, particularly for stilbenes, isoflavones, and flavanones, since few studies have associated their bioavailability with health effects. In addition, the biological effects of phenol metabolites derived from microbiota fermentation should be more extensively studied, since several data suggest their role in mediating the benefits of polyphenols.

Finally, since polyphenol bioavailability can be affected by food matrix components, specific strategies could be considered in order to increase their in vivo delivery (e.g., fermentation or exploiting the association among foods), or to protect them from degradation (e.g., microencapsulation).

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Review

Effects of Polyphenols on Insulin Resistance

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Abstract: Insulin resistance (IR) is apparent when tissues responsible for clearing glucose from the blood, such as adipose and muscle, do not respond properly to appropriate signals. IR is estimated based on fasting blood glucose and insulin, but some measures also incorporate an oral glucose challenge. Certain (poly)phenols, as supplements or in foods, can improve insulin resistance by several mechanisms including lowering postprandial glucose, modulating glucose transport, affecting insulin signalling pathways, and by protecting against damage to insulin-secreting pancreatic β-cells. As shown by intervention studies on volunteers, the most promising candidates for improving insulin resistance are (−)-epicatechin, (−)-epicatechin-containing foods and anthocyanins. It is possible that quercetin and phenolic acids may also be active, but data from intervention studies are mixed. Longer term and especially dose-response studies on mildly insulin resistant participants are required to establish the extent to which (poly)phenols and (poly)phenol-rich foods may improve insulin resistance in compromised groups.

Keywords: polyphenol; starch digestion; glucose; postprandial; GLUT4; akt; diabetes

1. Insulin Resistance

1.1. Characteristics of Insulin Resistance

Insulin resistance (IR) is a deceptively complex condition characterised by impaired insulin responsiveness in target tissues, causing the β-cells in the pancreas to continue to produce extra insulin, leading to eventual malfunction through oxidative stress. The lack of response of tissues to insulin results in a state of transient and unpredictable hyperglycaemia and hyperinsulinemia, together with an inflammatory signature that predisposes an individual to metabolic syndrome and type 2 diabetes (t2D) [1,2]. IR is a hallmark of obesity and of a sedentary lifestyle, driven by excess energy consumption, lack of exercise and certain genetic factors [3]. There is a complex interplay between several physiological processes that conspire together to facilitate development of IR, including an unbalance in lipid metabolism [4], dysbiosis [5,6], chronic inflammation, and misfiring of intracellular signalling pathways [7]. However, deciphering what is 'cause' of IR and what is 'effect' has proven much more difficult and conflicting opinions exist [4,8]. IR often precedes t2D, and the two conditions are inter-related. The course of t2D development is mainly characterized by declining β-cell function and worsening of IR [9].

In healthy individuals, the release of insulin from pancreatic β-cells drives cellular glucose uptake from the bloodstream into tissues via relocation of the glucose transporter solute carrier family 2, facilitated glucose transporter member 4, SLC2A4 (GLUT4) to the cell surface. This action removes elevated postprandial glucose from the blood, a normal process after eating carbohydrate. However, excess insulin production caused by a habitual high glycaemic index diet leads to elevated binding to the insulin receptor, driving fatty acid synthesis in addition to the normal glucose uptake. All of these

actions are dependent on a cascade of phosphorylation events, resulting in the activation of two pathways in particular: the phosphoinositide 3-kinase (PI3K) pathway and the mitogen activated protein (MAP) kinase pathway [10]. Dysregulation of the protein kinase B (akt) pathway most dramatically affects IR and can occur in adipose, liver and skeletal muscle, the three main tissue types that contribute to the phenomenon of IR. Obesity often leads to decreased akt activity by complex multifactorial mechanisms, which are incompletely understood [8]. However, decreased akt activity results in reduced GLUT4-mediated glucose uptake within adipose tissue and skeletal muscle [11].

1.2. Measurement of Insulin Resistance

Estimation of IR has been the subject of many publications and there are many models and opinions on the subject, which are beyond the scope of this review. The gold standard to which other methods are compared is the glucose or insulin clamp techniques [12], but these are time consuming, expensive, technically demanding and not practical for most hospitals, labs or other organisations. There are several less difficult ways to estimate IR, using various permutations of blood glucose and insulin concentrations, either fasting or after an oral glucose tolerance test (OGTT). One of the most commonly used is the homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR), requiring only fasting blood glucose and insulin measurement. It is often applied to large epidemiological studies as only these two values are required [13], and a subsequent modification is HOMA-β (also called HOMA2) using a modified equation but the same input parameters [14]. An alternative is the quantitative insulin sensitivity check index, abbreviated to QUICKI, illustrating the quality of scientific humour; this also only requires fasting plasma glucose and insulin concentrations [15]. The Matsuda index is one of the estimations that is derived from the fasting glucose and insulin concentrations, together with their values after an OGTT [16]. The diagnosis of IR can be affected by the method chosen, mostly based on variations in cut-off values [17]. These discrepancies demonstrate that IR is a gradual process and a continuum, with the cut-off value defined in a somewhat arbitrary manner. Triglycerides can also act as a surrogate marker of IR [18]. Anything that modulates one of these indices is considered to have the potential to modify IR, but a measured improvement will not necessarily change the diagnosis from "IR" to "non-IR", based on the truly continuous nature of the values. Measurement methods of IR have been reviewed and the choice of method is often determined by practicality, cost and ease of measurement in the clinic [19]. Insulin sensitivity is effectively the opposite of resistance; in simple terms, if low amounts of insulin are required to lower blood glucose, then insulin sensitivity is high and insulin resistance is low. The importance of IR as a risk factor for chronic diseases is well documented. For example, epidemiological studies have shown an association between IR as measured by HOMA-IR and risk of cardiovascular disease [20,21].

1.3. The Role of Chronic Inflammation in Insulin Resistance

Chronic inflammation is a well-characterised link between obesity and development of IR [2,3]. With obesity, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB)-driven pro-inflammatory cytokine secretion by adipocytes and immune cells is enhanced [22]. This leads to the recruitment and accumulation of adipose tissue macrophages, which produce more pro-inflammatory cytokines, including tumour necrosis factor (TNF)α and interleukin-6 (IL-6), that act as feed-forward mechanisms to exacerbate chronic inflammation, dysfunction of adipose tissue, decreased insulin sensitivity and IR [23–27]. The activation of inflammatory pathways has additionally been described in the liver [28,29] and muscle [10,29,30]. TNFα-signalling can also inhibit insulin receptor substrate-1 (IRS-1), attenuating its ability to propagate downstream insulin signalling [24]. Continuous over-consumption of energy can also lead to increased reactive oxygen species (ROS) production with endoplasmic reticulum stress, affecting the ability of β-cells to secrete insulin, and further attenuating inflammation and oxidative stress [31].

2. Factors that Affect IR

2.1. Macronutrient Intake

Long-term consumption of carbohydrate can affect IR, with significant reductions following intake of whole grain foods [32–36] and increases in IR with a habitual high glycaemic index diet [37]. The latter mediated changes in adiposity and central fat redistribution commonly observed in subjects with IR [38]. Concentrations of polyunsaturated fatty acids, such as n-3, have been found to be beneficial to insulin sensitivity in obese and overweight populations in some studies [39], but a Cochrane review found no overall significant effect of n-3 fatty acids on glycaemic control or fasting insulin [40]. Short term high fat diets (3 days) can induce transient IR [41,42], although results from longer time high fat diets, from 3 days to 4 weeks, were mixed and found either no change in insulin sensitivity [43,44] or a decrease [45]. Differences were also observed between ethnicities, with high fat, high energy diets having a greater effect in inducing IR in South Asians compared to Caucasians [46].

2.2. Lipid Metabolism

Lipid metabolism is disturbed in the IR state, partly due to the presence of increased circulating fatty acids and/or accumulation of lipids in the muscle and the liver [4] with activation of inflammatory pathways, inhibition of insulin receptor signalling and changes in the expression of genes that influence insulin action [47]. Hepatic IR is caused by the accumulation of lipids within the liver, rather than visceral fat accumulation [48,49]. Following a high fat diet or overfeeding, the accumulation of lipid outside of adipose depots can increase hepatic gluconeogenesis and disrupt GLUT4-mediated glucose transport in response to insulin in the muscle [4], leading to hyperglycaemia, stimulating β-cells to release further insulin and increase lipogenesis [8].

2.3. Gut Microbiota and Exercise

Obesity, a high-fat diet or both can lead to dysbiosis, an imbalance in the gut microbiota. This condition leads to increased intestinal permeability, with pro-inflammatory metabolites, such as liposaccharide (LPS) and other bioactive metabolites reaching the blood and ultimately acting on adipose and other insulin-responsive target tissues to promote IR [6]. LPS can act on the liver to promote triglyceride accumulation and secretion of pro-inflammatory cytokines leading to IR [6,50]. Structured mixed exercise can reduce IR [51], even in younger people [52], and reduce the risk of developing t2D.

3. Dietary (Poly)phenols

3.1. Sources and Intake

The nomenclature of (poly)phenols is complex and guidelines have been reported [53,54]. (Poly)phenols are made by plants for a variety of functions, including protection against stress, UV absorption and resistance to attack by pests. As a consequence, various classes of (poly)phenols are found in the diet and consumed on a regular basis. The different classes, structures and food sources have been extensively reported elsewhere and the reader is refereed to several comprehensive reviews [55,56] and to the phenol-explorer database [57].

3.2. Metabolism of (Poly)phenols

The absorption, metabolism, excretion and bioavailability of (poly)phenols has been reported in many reviews e.g., [55,58]. (Epi)catechins are absorbed in the small intestine, and reach the circulation mostly in a conjugated form [59]. Quercetin occurs as a conjugated form in plants and foods, and the attached sugar is removed before absorption, by lactase phloridzin hydrolase in the small intestine or by the gut microbiota [60]. Anthocyanins and procyanidins are poorly absorbed intact, but their catabolic breakdown products are well-absorbed [58]. Phenolic acids are absorbed, but bioavailability is

improved by the action of the gut microbiota [61]. In general, (poly)phenols must reach their target site in order to be active. To affect carbohydrate digestion and intestinal glucose absorption, which occur in the gut lumen, (poly)phenols do not need to be absorbed, but to affect other sites, such as muscle, adipose and cells, the (poly)phenol or its active catabolites must reach the circulation in order to exert activity on the tissue.

3.3. General Epidemiology on (Poly)Phenol-Rich Foods and IR/t2D Risk

Epidemiological and human intervention studies are considered here when (poly)phenols, or (poly)phenol-rich foods, have an effect on fasting glucose or fasting insulin, and hence affect IR. Consumption of a 'healthy diet' pattern, high in vegetables, fruit and wholegrains, and, therefore, (poly)phenols, can lower t2D risk by 14% [62]. A European, multicentre, case control study found that participants in the highest quintile of flavonoid intake, in particular flavanols and flavonols, had a 10% lower risk of developing t2D than those in the lowest quintile [63]. A meta-analysis of prospective studies of consumption of apples and pears, high in flavanols, found an inverse association between intake and t2D [64]. The long-term effects of coffee consumption are convincingly linked to reductions in t2D development [65,66] and decreases in IR [67], and a meta-analysis on epidemiological studies on anthocyanin-rich foods concluded that a higher intake of anthocyanins improved HOMA-IR through changes in fasting insulin [68].

3.4. Interventions Using Foods

3.4.1. Cocoa and (−)-Epicatechin

The consumption of cocoa flavanols, such as (−)-epicatechin, improves insulin sensitivity in both healthy and hypertensive populations. When comparing white and dark chocolate consumption over 15 days, HOMA-IR was significantly lower and QUICKI was significantly higher after the latter [69,70]. In elderly subjects with mild cognitive decline, 2 months of a drink containing flavanols improved IR [71], but consumption for 5 days in obese adults did not change fasting glucose or insulin [72]. In a systematic review and meta-analysis of randomised, controlled trials, it was concluded that flavanol-rich cocoa consumption decreased HOMA-IR [73]. In healthy adults consuming pure (−)-epicatechin for 1 month, HOMA-IR was improved through changes in fasting insulin with no change in fasting glucose [74].

3.4.2. Green Tea

Green tea contains high amounts of galloylated catechins, such as (−)-epigallocatechin gallate and (−)-epicatechin gallate. Data on green tea consumption and IR are not entirely consistent. In one meta-analysis on green tea, there was no change in IR and glycaemic control in t2D patients [75], whereas other meta-analyses have demonstrated improvements in fasting plasma blood glucose when green tea is consumed for 0.5 to 2 months [76,77].

3.4.3. Anthocyanins and Berries

Although conclusions from studies on anthocyanins are mixed, in general it appears that anthocyanins and anthocyanin-rich foods improve IR. In a systematic review of 19 randomised controlled trials, anthocyanin supplementation improved HOMA-IR [78], and three out of six studies showed a positive effect on glycaemic profile with consumption of berries [79]. Consumption of blueberry extracts, rich in anthocyanins and other (poly)phenols, improved HOMA-IR in patients with t2D but not in insulin resistant adults, and blueberry consumption improved IR in obese and insulin-resistant adults in one study but not in two other studies (summarised in [80]). In overweight and obese subjects, the consumption of proanthocyanidins and anthocyanins for 2 months prevented increases in IR induced by a fructose challenge [81]. In diabetic patients, consumption of anthocyanins for 6 months also reduced IR [82].

3.4.4. Quercetin and Onions

The effect of quercetin or quercetin-rich foods on IR in unclear owing to a limited number of studies. A systematic review concluded that quercetin for 2 months reduced fasting plasma glucose, but only at high doses [83]. Supplementation with quercetin-3-*O*-glucoside for 1 month to prehypertensive but otherwise healthy men showed no effect on IR [74], and supplementation with 500 mg quercetin for 1 month did not change fasting glucose in mildly hyperuricaemic men [84]. In breast cancer patients, yellow onions containing quercetin for 2 months resulted in a decrease in blood insulin and QUICKI, but this did not translate to a change in HOMA-IR nor HOMA-β [85]. It is notable that a systematic review on 13 animal studies concluded that quercetin dose-dependently lowered fasting blood glucose [86].

3.4.5. Phenolic Acids and Other Phenolic Compounds

The effect of consumption of coffee containing phenolic acids on IR is controversial, with studies reporting opposing conclusions. A trial for 8 weeks of up to 5 cups of coffee per day found no changes in insulin or glucose markers [87]. In a systematic review including six studies, green coffee extract lowered fasting blood glucose, but only higher doses were effective at improving HOMA-IR [88]. Phenolic compounds found in olive leaf, oleuropein and hydroxytyrosol, improved both the action of insulin and secretion from pancreatic β-cells following a 12-week supplementation in middle aged overweight men [89].

3.4.6. Stilbenes and Wine

Consumption of resveratrol and wine appear to have limited effects on IR, but the results are contradictory even in systematic reviews addressing similar criteria. Supplementation with the stilbene, resveratrol, unique to grapes and red wine, at much concentrations higher than in the fruit or in wine, attenuated fructose-induced oxidative stress and IR in first-degree relatives of type 2 diabetic patients and in obese individuals [81,90]. Red wine consumption for 2 weeks improved IR in people with t2D [91] but on the other hand there were no improvements in insulin sensitivity in obese subjects after 8 weeks of supplementation with (poly)phenols extracted from red wine [92]. A meta-analysis considering nine randomised intervention studies on t2D patients concluded that there was no effect of wine on fasting glucose and insulin [93], with a similar conclusion on t2D patients reached for pure resveratrol in one publication [94], but with a beneficial effect on HOMA-IR reported in an alternative systematic review on resveratrol [95]. Another systematic review concluded that resveratrol did affect fasting blood glucose levels, but only at high doses for >2.5 months [96] and in a systematic review on grape polyphenols, there was no apparent improvement of IR [97].

3.4.7. Hesperidin and Citrus Fruits

According to systematic review including six trials, hesperidin supplementation had no effect on fasting glucose, insulin, QUICKI nor HOMA-IR, despite positive indications from animal studies [98].

3.4.8. Pomegranate and Ellagitannins

In a systematic review, pomegranate intake did not show any improvements in glucose and insulin metabolism in healthy or compromised individuals [99], nor in patients with t2D [100].

3.4.9. (Poly)phenols from Nuts

Nuts contain a high quantity of fats, certain types of which may contribute to improving IR. In addition, nuts contain (poly)phenols, which could contribute to their ability to affect metabolic status. Pecans are particularly high in proanthocyanidins and ellagitannins, pistachios and almonds are high in proanthocyanidins, and walnuts are high in ellagitannins [101]. Consumption of pistachio nuts, according to 6 intervention studies, improved HOMA-IR through changes in fasting glucose [102],

but a systematic review on walnuts concluded that there was no effect on fasting blood glucose or other measures of IR [103]. Although there are fewer reported studies on humans, pecan and almond consumption seem to improve HOMA-IR [104,105] and almonds are also effective at reducing IR in t2D patients [106].

4. Mechanisms in the Pathway of Developing IR That May Be Affected by (Poly)phenols

In assessing the mechanism of (poly)phenols on insulin signalling and resistance, considerations need to be given to the use of the appropriate model, the physiological concentration of (poly)phenols in the right form, and the biochemical markers measured. Numerous publications report cell signalling changes after treatment with a high concentration of (poly)phenol, far above that which is physiologically relevant. It is also necessary to consider data from human interventions, and if no evidence of a beneficial effect is available, then it seems irrelevant to study mechanisms in vitro. Based on this reasoning, (−)-epicatechin is one of the best candidate (poly)phenols to affect IR. Data on quercetin in humans is quite limited. Resveratrol and hesperidin do not seem to affect IR in human interventions, but phenolic acids could be active. Anthocyanins seem effective in vivo in some studies, but since anthocyanins are poorly bioavailable, then it is most likely that the effects are due to anthocyanin catabolites, not the parent molecules. Even though some (poly)phenols are not active on IR as measured by fasting blood glucose and insulin, this does not mean that they do not affect metabolic disease risk. For example, there may be effects on blood pressure, cholesterol, uric acid or triglycerides, which would also affect cardiovascular disease and t2D risk.

There are some indications that lack of insulin responsiveness is temporary, and is reversible under the right circumstances [107]. IR transiently results from a constant accelerated formation of ROS in tissues, caused by obesity/chronic inflammation and high glucose/NADPH oxidase (NOX) in muscle and adipose. In the short term, the ROS are not enough to destroy the adipose or muscle cells, but put the metabolism off balance with impaired response to insulin owing to pathway disruption. However, ultimately the ROS stress will lead to the recruitment of pro-inflammatory cells, eventually causing permanent damage [108]. In response to the lack of insulin responsiveness, β-cells produce more and more insulin to try to elicit the clearance of glucose. This over-activity of the β-cells causes permanent damage and burnout, exacerbated by the presence of pro-inflammatory cytokines, in a highly complex mechanism [109]. Pancreatic β-cells have low levels of glutathione peroxidase and almost no catalase, and so are highly sensitive to ROS. Excessive prolonged ROS production can readily cause oxidative stress and lead to a deterioration of β-cell function [110]. Since the initial stages of insulin responsiveness are reversible, then there are several mechanisms by which (poly)phenols can improve the condition, which are briefly summarised below. In addition, the models used and mechanisms involved in protecting the β-cells from damage are summarised in Section 5 below.

4.1. Effects of (Poly)phenols on Starch Digestion

We and other have published reviews on the effects of (poly)phenols on postprandial glucose spikes through inhibition of carbohydrate digestion by enzyme inhibition [111,112] or by carbohydrate binding [113]. Starch is digested in the gut by α-amylases and α-glucosidases to produce glucose, which is then absorbed into the circulation. Glucose and fructose from dietary sucrose are also rapidly absorbed after hydrolysis by sucrase on the brush border of the small intestine enterocyte [114]. A high-carbohydrate meal consisting of starch and/or sugar will lead to a rapid increase of blood glucose, causing a release of insulin from β-cells to allow clearance of the glucose into tissues via GLUT4 activation by translocation [115]. Certain (poly)phenols can inhibit α-amylases and α-glucosidases and so slow down the rate of digestion, blunting glucose spikes. Since rapidly elevated glucose leads to formation of ROS in endothelial cells via NADPH oxidase 4 (NOX4) [116,117], then a smaller rise in glucose will give lower intracellular ROS in insulin-responsive cells. This leads to greater insulin sensitivity owing to less disruption of the pathways shown in Figure 1. The key requirements here are that the inhibition of α-amylase and α-glucosidase is strong enough to have an effect on blood

glucose postprandially. Acarbose is a drug used to manage diabetes and is a potent non-absorbed inhibitor of α-amylase and α-glucosidase, and long-term use leads to lowered risk of t2D [118]. To judge effectiveness, (poly)phenols can be compared to acarbose. Since (poly)phenols are consumed normally and continually in a habitual plant-rich diet, they do not have to be as potent as acarbose, but they must be sufficiently effective. Only a limited number of (poly)phenols are potent enough inhibitors to be effective in vivo, even though there have hundreds of publications on this activity. The most promising and potent (poly)phenols are punicalagin [119], epigallocatechin gallate [120,121] and quercetagetin [122], although the most pronounced effects are from foods [123], implying some interaction or synergy between (poly)phenols and other food components such as fibre.

4.2. *Effects of (Poly)phenols on Glucose Transport*

The inhibition by (poly)phenols on glucose transport is also a potential mechanism to lower postprandial blood glucose [124,125]. In order to exert a positive effect on insulin sensitivity and lower blood glucose after a meal, it would be necessary to attenuate glucose transporters in the gut to slow down absorption, but to stimulate glucose transport into tissues to exert an insulin-like effect. Clearly, it is unrealistic to expect the same compound to inhibit a transporter at one site and stimulate at another. There is some evidence that intact parent (poly)phenols, such as quercetin and green tea catechins can inhibit glucose uptake in the gut [126,127], and that (poly)phenol gut microbial metabolites could stimulate glucose uptake in tissues by through glucose transporter GLUT4- and PI3K-dependent mechanisms [128]. Further, quercetin increased GLUT4 translocation and akt signalling in mouse epididymis adipose tissues [129]. This dual effect of parent compounds and microbial products is a promising line of exploration and could conceivably lead to synergistic effects between the parent compound and its microbial degradation products.

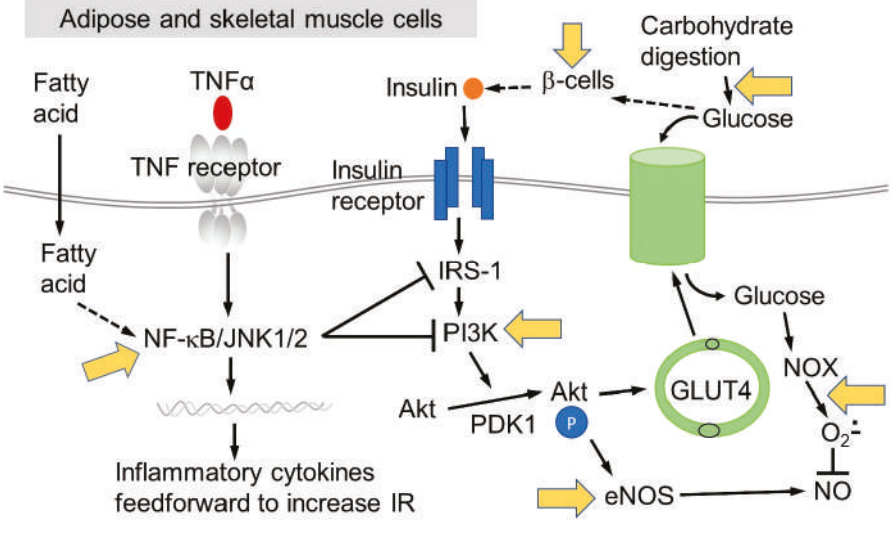

Figure 1. Simplified mechanism of glucose uptake by adipose or muscle tissue. Data combined from: [4,10,24,47,111,130]. Abbreviations defined in text, except for: JNK1/2, c-Jun N-terminal kinase 1/2; PDK1, pyruvate dehydrogenase kinase 1; IRS-1, insulin receptor substrate 1; PI3K, phosphoinositide 3-kinase; eNOS, endothelial nitric oxide synthase.

4.3. *Attenuation of Impaired Insulin Signalling and Pro-Inflammatory Pathways by (Poly)phenols*

(Poly)phenols can have multiple potential effects on the pathways by which cells take up glucose in response to insulin (Figure 1). Mechanistic information has been mostly derived from cultured cells

or animal models. Since any action on non-intestinal cells is mediated through the blood, then the active compound must teach the target tissue in sufficient concentration and in an active form [131]. There are many examples in the literature where cells have been treated with (poly)phenols and some molecular players in the insulin responsive pathway have been modulated. Here, only a selection are reported to illustrate the types of studies that have been done [132], where the action was observed at a reasonable concentration and the studies shown are certainly not exhaustive. In differentiated white adipocyte 3T3-L1 cells, (−)-epicatechin at relatively low concentrations dose-dependently inhibited the effects of tumour necrosis factor (TNF)-α-induced activation of mitogen activated protein kinase (MAPK), NF-kB, and AP-1 [133]. In adipocytes under inflammatory conditions where IR is impaired, quercetin restored GLUT4 translocation by beneficial regulation of IRS-1 function [134]. In rats given high fructose or mice given a high fat diet, (−)-epicatechin attenuated the negative effects of the dietary stress through NOX4, NF-kB, and extracellular signal-regulated protein kinase (ERK1/2) pathway modulation [135,136]. Ferulic acid dose-dependently improved HOMA-IR in rats fed a high fat, high carbohydrate and high fructose diet, at least partly through downregulation of NOX and inhibition of inflammatory cytokine production [137].

5. Cell Models for Protection of β-Cells against Oxidative Damage

Numerous insulin-secreting cell lines are used to understand more about the mechanism of developing IR. A majority of these release insulin in response to glucose, and have been obtained from rats, mice or humans. A summary is shown in Table 1. Of these, INS-1 cells obtained from rat pancreatic β-cells have been utilised in numerous studies with (poly)phenols. Other cells are also used to examine the mechanism of modulation of IR in tissues, including adipose and muscle tissue-derived cells.

Quercetin can protect INS-1 β-cells under stress conditions, potentiating glucose-induced insulin secretion [138], through activation of ERK1/2 [139]. At lower concentrations of glucose, quercetin can also stimulate insulin secretion via Ca^{2+} influx [140]. Under fructose-induced hyperinsulinaemia, quercetin can reduce insulin production by INS-1 β-cells and this correlates with decreased nuclear Foxo1 localization and reduced phosphorylation of akt [141]. (−)-Epicatechin at low concentration can improve glucose-stimulated insulin secretion in INS-1 cells impaired by saturated fatty acids [142], and at high concentrations, can reduce the production of glucose-induced ROS [142]. (−)-Epicatechin can also improve INS-1 cell viability and insulin secretion under chemically induced oxidative stress [143]. (−)-Epigallocatechin-3-gallate protects INS-1 cells against ethanol-induced apoptosis via the upregulation of the transcription factor NeuroD1, resulting in a decreased CCAAT-enhancer-binding protein (C/EBP) homologous protein transcription factor expression and decreased apoptosis [144]. Anthocyanins promote insulin secretion from β-cells in the presence of glucose [145], linked to intracellular Ca^{2+} signalling in insulin secretion [146]. Anthocyanins have also been shown to protect INS-1 cells from autophagic cell death induced by H_2O_2 stimulation [147]. Finally, ferulic acid stimulated insulin secretion in INS-1 β-cells, as measured by a rise in Ca^{2+} [148].

6. Conclusions

There are many studies examining the effect of (poly)phenols on insulin resistance in human intervention studies, in animal models and in cells. It can be concluded that (−)-epicatechin and (−)-epicatechin-rich foods such as cocoa can improve IR, and anthocyanins are also probably active. However, for other classes, there are still too many uncertainties to conclude that there is a protective effect. In addition, the mechanisms are still not clear, and there is a need for mechanistic studies using chronic low concentrations of relevant (poly)phenols. In addition, there are vast gaps in knowledge on the effect of (poly)phenol catabolites derived from the gut microbiota on the processes leading to IR. It seems that the (poly)phenols are a highly promising class of commonly consumed dietary compounds for improving IR, but more research is needed in order to give better dietary advice and improve the prospects of metabolically at-risk groups.

Table 1. Cell models used to study effects on pancreatic β-cells.

Name	Organism	Characteristics	Studies and Reference
INS-1	Rat	β-cell line secreting insulin in response to glucose. Line is stable with time, with high glucose-induced insulin secretion	Polysaccharide-induced insulin secretion [149] Lipotoxicity [150] Bacteria [151] Cytotoxic effects of mangostin [152] Glycogen metabolism [153] Effect of Indian culinary plants [154]
RIN	Rat	Exhibits glucose-stimulated insulin secretion	[155–157]
Min6	Mouse	Releases insulin in response to glucose, but high passage cells have impaired insulin secretion	[158] Establishment [159] Ginseng root-treated [160] Statin-treated [161] [162]
1.1B4	Human	Secretes low levels of insulin when glucose-stimulated	Cellular response to hyperglycaemia [163] [164–167]
EndoCβH1	Human	Secretes insulin in response to a glucose challenge, but slow growing and difficult to culture	Characterisation [168] Validity in drug screening against mouse model [169] [170,171] Further studies listed in review [172]

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