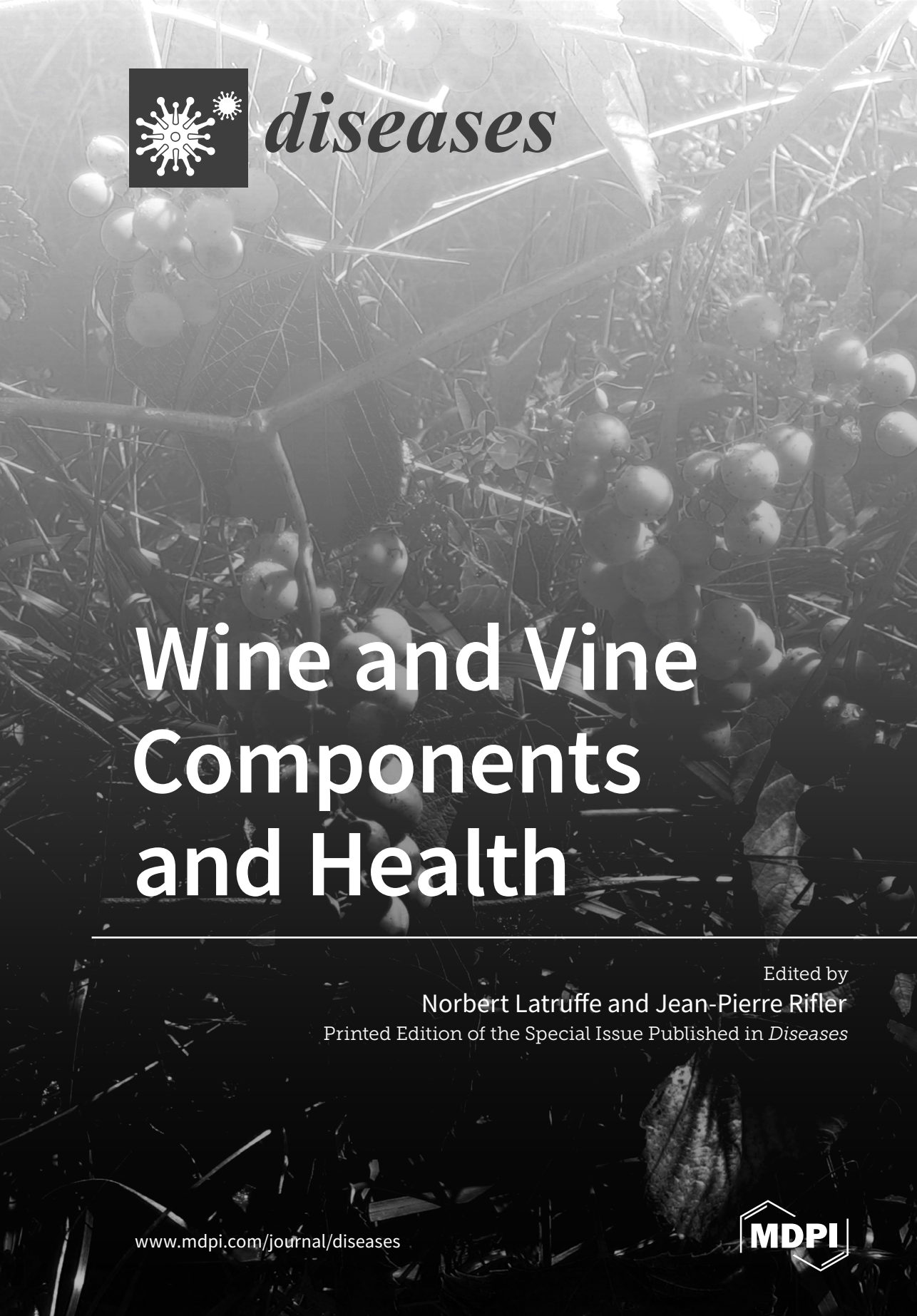

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Wine and Vine Components and Health

Edited by
Norbert Latruffe and Jean-Pierre Rifler

Printed Edition of the Special Issue Published in *Diseases*

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Special Issue Editors

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Cover image courtesy of Norbert Latruffe and Jean-Pierre Rifler.

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About the Special Issue Editors

Norbert Latruff (http://bioperoxil.u-bourgogne.fr/) obtained his Ph.D. in 1977, and in 1989, was appointed full Professor in Biochemistry at the University of Burgundy-Dijon, France, where he was head of the Laboratory of Molecular and Cellular Biology until 2006. Following that, he was in charge of the team of Biochemistry of Metabolism and Nutrition in the INSERM research center, UMR 866 of Dijon until the end of 2011. Since 2013, Latruffe has been senior Professor at the Laboratory of Biochemistry at the Faculty of Life Science. In 1998, he has been addressing new challenges regarding the preventative role of vine polyphenols, especially resveratrol, against age-related pathologies such as cancer and inflammatory cardiovascular disease. He was one of the first to explore resveratrol transport and metabolism (2004) and its pro-apoptotic properties (2004), and discovered a new resveratrol signaling pathway through microRNA modulation (2010). In 2014, he demonstrated the preventive effect of wine polyphenols towards colon cancer in mice models. Latruffe has organized several and workshops on wine and health. Latruffe has recently served as Editor and Co-editor of two Special Issues of *Molecules*: Natural Products and Inflammation (2016) and Improvement of Resveratrol Efficacy (2017). To date, Latruffe has published nearly 170 international papers and presented over 140 invited lectures. In 2017, he managed the a book deal on an edition covering wine and the Mediterranean diet (EUD editor, Dijon). Latruffe is an expert or past expert of several evaluation councils, and is a member of the orientation council of the prestigious UNESCO Chair "heritage and traditions of wine". He has also been awarded several distinctions.

Jean-Pierre Rifler is emergency physician at the Haute Côte d'Or hospital center, F-21350, France (https://etablissements.fhf.fr/annuaire/hopital-fiche.php?id_struct=473) and is passionate about wine. After his obtained a diploma of Oenolog technician in 1988, he prepared his medical doctoral thesis in 1994 on wine and health, especially on the cardiovascular protection of red wine polyphenols. Since this date and in parallel to his professional activities, he has collaborated with scientists in Burgundy with the aim of promoting knowledge regarding the beneficial effects of a regular and moderate consumption of wine. He has managed the theses of medical doctors and set up clinical protocols to validate secondary prevention for post-infarcted patients by wine in 2012. This important work has been published in collaboration with Prof. Norbert Latruffe, co-Guest Editor of this Special Issue "A Moderate Red Wine Intake Improves Blood Lipid Parameters and Erythrocyte Membrane Fluidity in Post-myocardial Infarct Patients" by Rifler JP., et al., Molecular Nutrition and Food Research 2011. vol. 55 pp. 1–7. Dr. Jean-Pierre Rifler has also published numerous specialized papers and is periodically invited in congresses and organizations such as in WAC (Wine Active Compounds), Beaune 2011; or 5è ICPH (International Conference on Polyphenols and Health), Sitges (Barcelona), 2011. He is co-founder of the Mediterranean Nutrition and Health Association which organized a colloquium in Hyères (Provence) in 2016 on Wine, Mediterranean Nutrition, and Health. Dr. Jean-Pierre Rifler is also well known for having initiated the French program on heart defibrillators in public spaces to save lives.

Preface to "Wine and Vine Components and Health"

In terms of biochemical mechanisms, vine, like other plants, produces numerous non-energy compounds called secondary metabolites (e.g., flavonoids, polyphenols), in order to adapt their defenses against often unfavorable environments (biotic and non-biotic stresses). Interestingly, in humans and in the animals kingdom, these microconstituents provide similar valuable bioactive properties for essential cell and physiological functions (signaling, gene regulation, prevention of acquired or infectious disease, etc.). These compounds have been selected through evolution and are generally preserved in all living organisms. For instance, resveratrol, that plays an essential role in vine plants as an elicitor of natural defenses, has been shown to be a protector of health in humans. It can delay, or even block, the appearance of predominant diseases, such as atherosclerosis, by protecting low-density lipoproteins from the oxidation, in addition to positive effects on diabetes and cancer.

Grape, both fresh or dried, is a widely consumed fruit by large human populations, as are its byproducts, including grape juice and wine and even extracts of vine leaves and shoots. Grape products contain vast and highly varied quantities of polyphenols as a protective micronutrient. Wine provides unique polyphenols, for instance, resveratrol, procyanidins, and monophenols such as hydroxytyrosol and tyrosol. Research supports the idea that wine—a natural biological product—if consumed regularly and not in excess, can act preventively. In addition to eliciting its more well-known activities against vascular diseases (illustrated by the so-called French paradox), the moderation consumption of wine may also prevent infections, decrease inflammation, and delay neurodegenerative diseases. Regarding cancer, the question remains open.

Despite the huge amount of data on this topic, there are still gray areas and incomplete knowledge. This is why the objective of this Special Issue is to promote a better view of wine, especially through policy makers, the medical world, and the vectors of image, in order to explain the rationalization and philosophy with respect to ethics and public health.

Norbert Latruffe, Jean-Pierre Rifler
Special Issue Editors

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Editorial

Special Issue: Wine and Vine Components and Health

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There is much literature on the topic of wine and health dating back to the days of Hippocrates, and it is believed that there are unlimited varieties of wine, allowing the association of senses, nutrition, and hedonism. The history of vine and wine has lasted for at least 7000 years (Latruffe, 2018 [1]). *Vitis* is an adaptable plant, thanks to a large variety of strains; wine is an alchemy with unique properties, a rich and original composition in terms of polyphenols and well-known antioxidants (Figure 1, see below). This explains why wine and health are closely linked to nutrition (Latruffe, 2017 [2]).

Figure 1. Figure of the authors.

In terms of biochemical mechanisms, vines like other plants produce numerous non-energy compounds, called secondary metabolites (e.g., flavonoids, polyphenols), in order to adapt their defenses against an often unfavorable environment (biotic and non-biotic stresses). Interestingly, in humans and in the animal kingdom these microconstituents provide similar valuable bioactive properties for essential cell and physiological function (signaling, gene regulation, prevention of acquired or infectious disease, etc.). These compounds have been selected through evolution and are generally preserved in all living beings. For instance, resveratrol that plays an essential role in vine plants as elicitor of natural defenses has been shown to be a protector of health in humans. It can delay, or even block, the appearance of predominant diseases such as atherosclerosis by protecting low-density lipoproteins from oxidation, but also diabetes and cancer.

The grape, fresh or dried, is a fruit widely consumed by large human populations, as well as its by-products such as grape juice and wine. Some even use vine leaf extracts and vine shoots. Grapes contain vast and highly varied quantities of polyphenols as a protective micronutrient. Wine provides unique polyphenols—for instance, resveratrol, procyanidines, and monophenols such as hydroxytyrosol and tyrosol. Research supports the idea that wine, which is a natural biological product, if consumed regularly but without excess, possesses preventive properties, not only having its well-known properties against vascular diseases (illustrated by the so-called French paradox) but also possibly preventing infections, decreasing inflammation, and delaying neurodegenerative diseases. The question with respect to cancer is still open.

Despite the huge amount of data on this topic, gray areas still remain and knowledge is incomplete. That is why the objective of this issue is to present a better view of wine, especially through policy makers, the medical world, and the vectors of image in order to explain the justification and the philosophy of wine with respect to ethics and public health.

This Special Issue of the journal *Diseases* focuses on wine and vine components and health and includes the effects of wine on human physiology (cardiovascular diseases, aged-linked disorders, etc.); the effects of polyphenols as wine antioxidants and as signaling molecules; and, from a humanity point of view, the tasting properties of wine.

We edited four primary articles and five reviews providing new data and new concepts related to the following keywords: antioxidant capacity, wine, vine, and grape components, including ethanol and polyphenols such as resveratrol, and flavonoids; their metabolism and their effect on pathologies such as aging, longevity, vascular diseases, diabetes, cancer, inflammation, allergies, neurodegeneration, among others. The paper entitled "Is a Meal without Wine Good for Health?" by Jean-Pierre Rifler [3] has been selected as the issue cover.

The new findings from original articles are as follows.

Concerning innovative technology, a paper reports on an Electrochemical Method for Evaluating Antioxidant Capacity of Wines, called PAOT ("Pouvoir Anti-oxydant Total"). Using this method, the authors found that the total antioxidant activity was almost seven-fold higher in red wines when compared to rosé and white wines from the commercial market. Winemakers can use PAOT to evaluate the antioxidant activity of wine during the winemaking process (Pincemail et al., [4]).

A case control study was carried out by Boronat et al. [5] on wine and olive oil phenolic compounds and metabolism in humans. They studied the metabolism of resveratrol (from red wine), and of tyrosol and of hydroxytyrosol (from red wine and from extra virgin olive oil) and found an increase in urinary tyrosol and hydroxytyrosol from a combination of red wine and extra virgin olive oil intake, whereas resveratrol remained identical as red wine intake only.

With the aim of slowing neurodegeneration associated with aging, especially Alzheimer's disease and Parkinson's disease, the effects of resveratrol and other Mediterranean diet-associated polyphenols have been studied with respect to neuronal differentiation (Namsi et al., [6]). Interestingly, they found that resveratrol and apigenin can induce cultured cell neuronal differentiation.

A preclinical study on spontaneously hypertensive rats (SHR) was performed to analyze the remaining potential of grape by-products from various red wine cultivars (Rasines-Perea et al.; [7]). Extracts used from grenache, syrah, and alicante cultivars presented a "rebound effect" on systolic blood pressure, whereas the other extracts (carignan, mourvedre, etc.) showed no significant changes.

Review papers presented current knowledge on different subjects featured in the Special Issue.

Tanaka et al. [8], reported on the potential beneficial effects of wine flavonoids on allergic disease models, but the evidence in humans is limited to allergic rhinitis and respiratory allergy.

Vervandier-Fasseur's group [9] selected the synthesis of innovative *trans*-resveratrol derivative procedures, in order to increase its solubility in water and pharmacological activities toward cell targets.

The potential effects of polyphenol extracts from red wine and grapevine on cancers have been summarized by Amor et al. [10]. They discuss how the polyphenolic composition of red wine may influence its chemopreventive properties.

Pavlidou et al. [11] compared wine to an aspiring agent in promoting longevity and preventing chronic diseases. They especially highlight the beneficial role of red wine against oxidative stress and in favor of desirable gut bacteria, so-called microbiota, where some promising studies are pending.

After having recalled that wine is the elixir that, by design and over millennia, has acted as a pharmacopeia that has enabled people to heal and prosper on the planet, Rifler [3] pointed out the characteristics of wine drinking linked to religion, culture, civilization, and the manner of eating (insisting on the Cretan and Okinawa diets). He finishes with the following message:"Moderate drinking gives a protection for diseases and a longevity potential. In conclusion, let us drink fewer, but drink better, to live older."

This Special Issue of *Diseases* focusing on the effects that wine and vine components have on health allows us to publish new findings on antioxidant capacity measurement using innovative technology, on the metabolism of polyphenols with respect to humans, on the induction of neuron differentiation in cell models by resveratrol, and on the regulatory effect of hypertension in animals by some wine by-products. On the other hand, reviews make statements on wine polyphenols in connection with allergy/inflammation, with cancer, with intestine microflora, and with diet. Finally, we learn about perspectives opened by new resveratrol derivatives to fight low bio-availability of the parent molecule.

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

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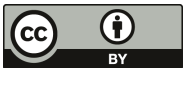

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Article

PAOT-Liquid® Technology: An Easy Electrochemical Method for Evaluating Antioxidant Capacity of Wines

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Abstract: Polyphenol compounds present in high quantity in wines are well-known to have potent cardio-protective properties through several biological mechanisms including antioxidant activity. A large number of methods have been developed for evaluating the antioxidant capacity of food matrices. Most of them have, however, the disadvantage of being time consuming and require specific analytical protocols and devices. In the present study, we present the electrochemical PAOT (Pouvoir Antioxydant Total)-Liquid® Technology which can be easily used by winemakers for evaluating antioxidant activity of wine during all steps of making process. The methodology is based on the measurement of electric potential variation resulting from chemical reactions between wine polyphenols and a free radical mediator $M^{\bullet}$ as source of oxidants. Total antioxidant activity as estimated by the PAOT-Liquid® activity was 6.8 fold higher in red wines (n = 14) when compared to rosé (n = 3) and white (n = 3) wines bought in a commercial market. Moreover, PAOT-Liquid® activity was highly correlated with total polyphenols content (TPC) of all wines (r = 0.9540, $p < 0.0001$) and the classical DPPH (2,2-diphenyl-1-picryhydrazyl) assay which is often used for evaluating antioxidant capacity of food matrices (r = 0.9102, $p < 0.0001$).

Keywords: polyphenols; antioxidant capacity; electrochemical technology; wine

1. Introduction

A large number of studies have evidenced that oxidative stress plays a key role in the development of several pathologies including cardiovascular, neurological and inflammatory diseases, cancer and diabetes [1]. Jones has defined oxidative stress as an imbalance between reactive oxygen species or ROS (including free radical and non-free radical species) and antioxidants in favor of the formers, leading to a disruption of the redox signaling and/or molecular damage to lipids, proteins and DNA [2]. Among antioxidants, a large amount of interest has been given to the large family of polyphenols which can be divided into lignans, stilbenes, tannins, phenolic acids (benzoic and cinnamic acids derivatives) and flavonoids (flavonols, flavanones, flavones, flavanols or catechins, anthocyans and isoflavones). The potential health benefits of polyphenols were first highlighted by the Zutphen's study, which evidenced an inverse relationship between intake in diet flavonoids and the risk of developing cardiovascular diseases [3]. Moreover, the adhesion to the Mediterranean diet known for its richness in polyphenols is well recognized to be a guarantee of good cardiovascular health [4,5]. The capacity of polyphenols to regulate the arterial blood pressure by maintaining a good endothelium

health [6] but also their ability to stimulate genes coding for the expression of antioxidant enzymes through Keap1/NrF2/ARE activation [7] have, among other mechanisms, prime places for explaining such cardio-protective effects.

Repartition of polyphenols in natural foods is as follows: fruits (41%), fresh vegetables (11%), dry vegetables (8%) and processed products such as fruit juices, cocoa, coffee, green tea, olive oil but also red wine (33%). Over the past decade, the health effects of moderate red wine consumption (125 mL glass) by reducing risk of developing cancer and cardiovascular diseases have been the matter of many studies (for a review see references [8,9]). However, the wine polyphenol composition and, therefore, its antioxidant capacity can be strongly affected by winemaking techniques and oenological practices [10]. In the present paper, we present the PAOT-Liquid® technology which is able to measure the total antioxidant capacity of wine, and indirectly their total polyphenol content (TPC), thanks to a fast electrochemical application.

2. Material and Methods

Antioxidants gallic acid (GA), catechin (C), epicatechin (EC), epigallocatechin gallate (EGCG), epigallocatechin (EGC), gallocatechin (GC), myricetin, quercetin, kaempherol, naringin, hesperdin methyl calcone, cyanidin chloride, delphinidin chloride, pelargordin chloride, free radical 2,2-diphenyl-1-picryhydrazyl (DPPH) and Trolox (T) were all purchased from Sigma, Nancy and Lyon, France. Folin's reagent, methanol and sodium carbonate have been supplied by WWR International, Fontenay-sous-Bois, France. Wines including 14 red, 3 rosé and 3 white produced in five different countries have been bought in a commercial market in Belgium.

2.1. Total Polyphenols Content (TPC)

Total polyphenols content was determined by the Folin–Ciocalteu method [11]. Appropriately diluted extract (3.6 mL) was mixed with 0.2 mL Folin–Ciocalteu reagent and 3 min later, 0.8 mL sodium carbonate (20% w/v) was added. The mixture was heated at 100 °C for 1 min. After cooling, the absorbance at 750 nm was measured. Using gallic acid (GA) as a standard, results were expressed as mg gallic acid equivalents/par liter (GAE) L^{-1}.

2.2. DPPH Assay

Antioxidant capacity of wines was determined by the DPPH (free radical 2,2-diphenyl-1-picryhydrazyl) assay as initially described by Tadolini et al. [12]. All complete details about the protocol were provided in a previous paper of us [13]. Trolox (T) was used as standard and the antioxidant capacity was expressed in µmol Trolox equivalent/liter (TE) L^{-1}.

2.3. PAOT-Liquid® Assay

PAOT (Pouvoir Antioxydant Total) Liquid® Technology is a method allowing total antioxidant capacity determination in various matrices, such as raw materials and processed food products, cosmetic and medicinal preparations, biological fluids or plant extracts [14]. The PAOT Liquid® Technology is actually the subject of a patent application filing (patent FR1871986; 11.28.2018). Thanks to the robust and easily transportable device shown on Figure 1, the measurement was carried out in a reaction medium (1 mL physiological solution at pH ranging from 6.7 to 7.2, temperature 24–27 °C) containing a molecule in a free radical state called mediator ($M^{\bullet}$). Two microelectrodes, one being the working electrode and the second one the reference electrode, were then immersed in the medium. After addition of 20 µL of pure antioxidants (1 mM final) or wine samples, PAOT-liquid® activity was estimated by registering electrochemical potential modifications in the reaction medium (due to changes in the concentration of oxidized/reduced forms of the mediator $M^{\bullet}$ during reaction with antioxidants as AOX (oxidized mediator $M^{\bullet}$ + AOX -→ reduced mediator M + oxidized AOX) [15].

Figure 2 shows the typical curve of the electrochemical potential registration after 10 min of interaction of AOX or wine simples with mediator M•. Results were calculated according to the following formula:

$$\text{antioxidant activity} = \left(\frac{\left(EP_{product\ 10} - EP_{control\ 0} \right)}{EP_{control\ 0}} \right) \times 100\%, \quad (1)$$

where $EP_{control\ 0}$ was the electrochemical potential at time 0 and $EP_{product\ 10}$ the electrochemical potential obtained after 10 min registration in presence of tested antioxidants or wine samples. Gallic acid was used as a standard and results were expressed as mg gallic acid equivalents (GAE) L^{-1}.

Figure 1. Photography of the PAOT-Liquid® Technology device showing both reference and working microelectrodes immersed in the reaction medium containing free radical mediator M• and antioxidants or wines samples.

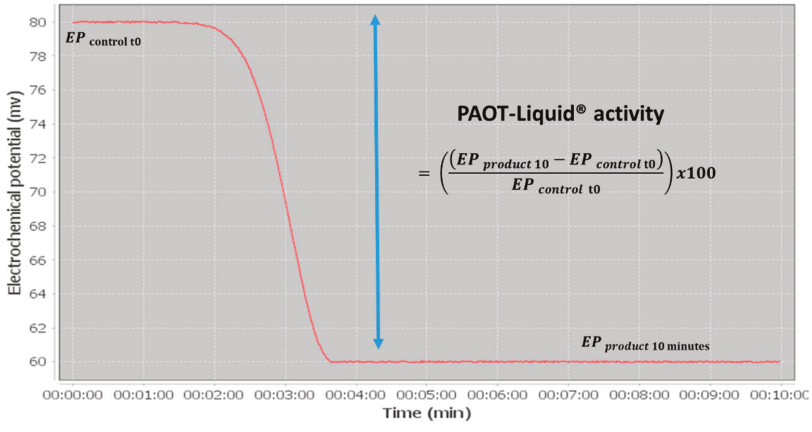

Figure 2. Kinetic curve of electrochemical potential changes during reaction of antioxidants or wines samples with the free radical mediator M•.

3. Results

Table 1 summarizes the characteristics of all tested wines (14 red, 3 rosé and 3 white) produced in different countries (France, Italy, South Africa, Chili and South Australia).

Table 1. Characteristics of tested wines bought in a Belgian commercial market.

Number	Color	Region/Country	Name	Vintage	Year
1	red	Beaujolais/France	Moulin à vent	Gamay	2015
2	red	Cachapoal Valley/Chili	La Capitana	Merlot	2014
3	red	Bordeaux/France	Château Tuilerie Pages	Cabernet Franc, Merlot, Cabernet Sauvignon	2014
4	red	Bordeaux/France	Château la Tuilerie Graves	Merlot, Cabernet Sauvignon	2016
5	red	Corbières/France	Château Prat de Cest	Syrah, Grenache, Mourvedre	2015
6	red	Barossa Valley/South Australia	Lindeman's Bin 50	Shiraz	2017
7	red	Mendoza/Argentina	Trivento	Malbec	2017
8	red	Bardolino/Italy	Giovanni Righetti	Corvina, Rondinella, Molinari	2017
9	red	Saint-Chinian/France	Valdorb rouge	Syrah, Grenache, Carignan	2017
10	red	Colchagua Valley/Chili	Koyle Reserva	Cabernet Sauvignon	2014
11	red	Western Cape/South Africa	Baie Cap	Pinotage	2017
12	red	Bourgogne/France	La chance du Roy	Gamay, Pinot Noir	2015
13	red	Minervois, France	L'aigle de Minerve	Carignan, Syrah, Grenache, Mourvedre	2016
14	red	Côtes du Rhône Villages/France	Côtes du Rhône villages	Grenache/Syrah	2016
15	rosé	Pays d'Oc/France	Syrah Rosé	Syrah rosé	2016
16	rosé	Pays d'Oc/France	Vin Gris	Cinsault, Syrah, Carignan, Grenache	2017
17	rosé	Corse/France	La Petite Paillote	Niellucciu, Sciaccarellu	2017
18	white	Pays d'Oc/France	Vent Marin	Chardonnay	2016
19	white	Val de Loire/France	Sauvignon de Touraine	Sauvignon Blanc	2017
20	white	Corse/France	La petite Paillote	Vermentino	2017

Table 2 describes the PAOT-Liquid® activity of main polyphenols, more particularly those of the flavonoid family, which can be found in wines. Tested at a concentration of 1 mM, myricetin belonging to the flavonol family exhibited the highest PAOT-Liquid® activity (677.78 mg (GAE) L^{-1}) when compared to quercetin (560.4 mg (GAE) L^{-1}) and kaempferol (404.56 mg (GAE) L^{-1}). In the anthocyanins family, cyanidin had the best score (512.54 mg (GAE) L^{-1}) in front of delphinidin and pelargordinin. Both EC (730.2 mg (GAE) L^{-1}) and EGCG (613.11 mg (GAE) L^{-1}) from the favano-3-ol subgroup were among all tested molecules those having the highest antioxidant capacity. For comparison, Trolox which is the antioxidant reference used in most in vitro assays, had only a value of 544.16 mg (GAE) L^{-1}. At least, both naringin (53.28 mg (GAE) L^{-1}) and hesperidin methyl calcone (51.85 mg (GAE) L^{-1}) from the flavanone group presented a score which was largely below those of all other tested flavonoids.

Table 2. PAOT-Liquid® activity of several flavonoids, the major subclass of polyphenols family. Comparison with Trolox used as reference antioxidant in the DPPH assay.

	PAOT-Liquid® Assay mg (GAE) L^{-1}
Flavano-3-ol Family	
Catechin	504.56 ± 45.58
Epicatechin (EC)	730.2 ± 93.73
Gallocatechin (GC)	431.05 ± 35.61
Epigallocatechin (EGC)	545.58 ± 45.87
Epigallocatechin gallate (EGCG)	613.11 ± 0.57
Flavonol Family	
Kaempferol	404.56 ± 55.27
Quercetin	560.4 ± 0.85
Myricetin	677.78 ± 7.41
Flavanone Family	
Hesperdin methyl chalcone	51.85 ± 0.57
Naringin	53.28 ± 0.28
Anthocyanidins Family	
Pelargonidin Chloride	284.33 ± 3.42
Delphinidin Chloride	340.74 ± 69.23
Cyanidin Chloride	512.54 ± 5.13
Other	
Trolox	544.16 ± 16.81

As shown in Table 3, the highest TPC (mean value: 1789 ± 367 mg (GAE) L^{-1}) was clearly found in red wines when compared to rosé (mean value: 265 ± 65 (GAE) L^{-1}) and white (mean value: 221 ± 28 mg (GAE) L^{-1}) wines. As suggested daily allowance in total polyphenols is around 1000 mg [16], the consumption of 125 mL glass of red wine, therefore, meanly affords 223 mg of TP. A large heterogeneity was, however, observed in red wines since values may vary from 1278 (wine 12) to 2349 mg (GAE) L^{-1} (wine 3). A total of 5/14 red wines had a TPC higher than 2000 mg (GAE) L^{-1} (wines 1, 3, 4, 10 and 13). Three of them (3, 4, 13) were multi-varietal while the two other ones were mono-varietal (1, 10). By contrast, 9/14 wines (2, 5, 6, 7, 8, 9, 11, 12, 14,) had values between 1278 and 2000 mg (GAE) L^{-1}. Six of them (2, 6, 7, 11, 12, 14) were mono- or bi-varietal and three multi-varietal (5, 8, 9). Statistical analysis revealed, however, that there was not significant difference between the mean value in TPC of mono or bi and multi varietal wines (1733 ± 125.6 mg (GAE) L^{-1}, $n = 8$ vs. 1864 ± 164.5 mg (GAE) L^{-1}, $n = 6$; $p = 0.57$).

Table 3. Total polyphenol content (TPC) in tested wines and their antioxidant capacity as assessed by DPPH method and PAOT-Liquid® Technology.

Number	Region/Country	TPC mg (GAE) L^{-1}	DPPH Assay μM (TE) L^{-1}	PAOT-Liquid® Assay mg (GAE) mg L^{-1}
Red wines				
1	Beaujolais/France	2129 ± 17.9	3119 ± 47.7	1067.5 ± 17.86
2	Cachapoal Valley/Chili	1545 ± 40.1	2628 ± 24.9	908.02 ± 39.13
3	Bordeaux/France	2349 ± 18.2	3732 ± 32.6	1267.39 ± 30.2
4	Bordeaux/France	2253 ± 9.7	3773 ± 72.9	1180.21 ± 2.98
5	Corbières/France	1450 ± 20.3	2738 ± 65.3	757.03 ± 11.91
6	Barossa Valley/South Australia	1323 ± 12.8	3082 ± 51.3	1054.74 ± 17.86
7	Mendoza/Argentina	1603 ± 14.68	3168 ± 32.7	878.24 ± 26.79
8	Bardolino/Italy	1511 ± 11.8	1474 ± 11.0	846.35 ± 17.86
9	Saint-Chinian/France	1563 ± 24.9	2874 ± 44.8	950.55 ± 26.79
10	Colchagua Valley/Chili	2239 ± 20.8	4219 ± 64.6	1280.15 ± 6.34
11	Western Cape/South Africa	1915 ± 17.5	2395 ± 20.1	942.04 ± 2.98
12	Bourgogne/France	1278 ± 41.5	1240 ± 4.5	1086.64 ± 8.93
13	Minervois, France	2060 ± 8.8	3912 ± 63.5	959.05 ± 32.75
14	Côtes du Rhône Villages/France	1831 ± 37.8	3065 ± 57.2	1088.77 ± 17.86
mean		1789	2958	1016.47
SD		367	854	153.11

Figure 3 evidences that there was a strong positive and significant correlation (r = 0.9540, $p < 0.0001$) between TPC and PAOT-Liquid® activity. The deep shift between red wines and rosé and white ones was confirmed. Among red wines, two different groups were identified as for TPC: wines 1, 3, 4, 10, 11, 13, 14 vs. wines 2, 5, 6, 7, 8, 9, 12. As shown on Figures 4 and 5, similar correlations were also evidenced when comparing PAOT-Liquid® activity and DPPH assay (r = 0.9036, $p < 0.0001$) or TPC and DPPH assay (r = 0.9417, $p < 0.0001$).

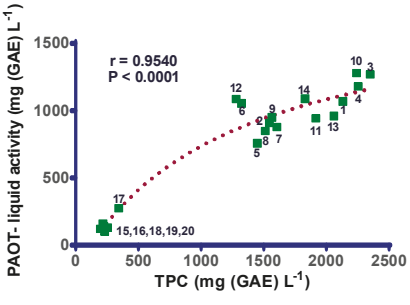

Figure 3. Correlation between TPC (total polyphenols content) and PAOT-Liquid® activity in red ($n = 14$), rosé ($n = 3$) and white wines ($n = 3$) bought in a Belgian commercial market.

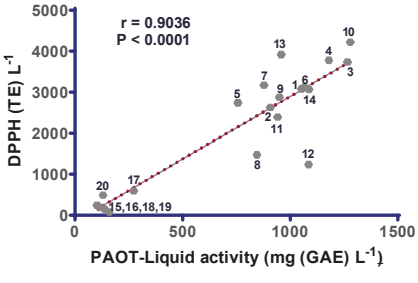

Figure 4. Correlation between PAOT-Liquid® activity and DPPH assay in red ($n = 14$), rosé ($n = 3$) and white wines ($n = 3$) bought in a Belgian commercial market.

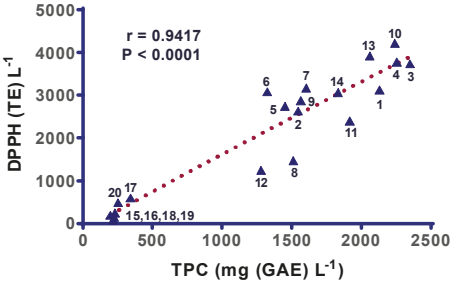

Figure 5. Correlation between TPC (total polyphenols content) and antioxidant capacity as assessed by DPPH assay in red (n = 14), rosé (n = 3) and white wines (n = 3) bought in a Belgian commercial market.

4. Discussion

A large number of methods have been developed to determine the in vitro antioxidant capacity of food matrices. They include two major groups: assays based on single electron transfer reaction (SET), in which the redox reaction between the antioxidant and the oxidant is measured by the change in the oxidant's color, as an indicator of the end of the reaction; and assays based on hydrogen atom transfer reaction (HAT), in which there is a competitive reaction between the antioxidant and the substrate (probe) for the free radicals. SET methods are Trolox Equivalent Antioxidant Capacity (TEAC) assay, Ferric Reducing Ability (FRAP) assay, Copper Reduction (CUPRAC) assay, and, finally, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assay which is the most popular. HAT assays include the crocin bleaching assay, the total peroxyl radical trapping antioxidant parameter (TRAP) assay, and overall, the Oxygen Radical Absorbance Capacity (ORAC) assay. Advantages and disadvantages of all these methods have been discussed in detail in a previous paper of us [17]. The PAOT-Liquid® Technology can be classified in the SET category since this electrochemical assay directly estimates the antioxidant capacity via the electric potential shift due to changes in the concentration of oxidized/reduced forms of the free radical mediator ($M^{\bullet}$) during reaction with antioxidants. Moreover, the use of microelectrodes for registering current changes from reaction between the oxidant mediator $M^{\bullet}$ and antioxidants rendered the method very sensitive.

As shown in Table 2, the PAOT-Liquid® Technology was perfectly able to evaluate the antioxidant capacity of molecules present in wines such as polyphenols from the flavonoids family. The relationship between PAOT-Liquid® activity and the structure of these compounds can be even evidenced. The basal chemical structure for all flavonoids is constituted of a benzene A ring linked to an oxidized heterocyclic C ring substituted in position 2 by another benzene B ring. In the flavanone family, two phenolic (–OH) groups are present on ring A in positions 5 and 7, one on the ring C in position 4 while ring B is respectively substituted by 1, 2 and 3 –OH groups respectively in case of kaempferol (position 4′), quercetin (positions 3′ and 4′) and myricetin (positions 3′, 4′ and 5′). Table 2 shows that the PAOT-Liquid® activity logically increased with the number of antioxidant –OH groups on ring B, myricetin having so the highest value in front of quercetin and kaempferol.

In the flavanol-3-ol family, benzene A rings possess two –OH groups on positions 5 and 7 while one –OH group is present on the heterocycle C ring on position 3. Ring B is substituted with 2 –OH groups in case of catechin and its isomer epicatechin (EC) on positions 4′ and 5′. Gallocatechin (GC) and epigallocatechin (EGC) have another –OH group on position 3′. When compared to GC, the chemical structure of epigallocatechingallate (EGCG) has the –OH group on the heterocyclic C ring substituted by a gallate group constituted of a benzene ring having 3 –OH groups. Due its large number of –OH groups (n = 9), EGCG has, as expected, one of the highest PAOT-Liquid® activity. It is instructive to note that both isomers of catechin and epicatechin have a higher antioxidant activity than the original form.

In the anthocyanins family, benzene ring A with two –OH groups on positions 5 and 7 is linked to a flavylium cation having a –OH group in position 3 and in position 2 by the benzene B ring. In case of pelagornidin, cyanidin and delphinidin, three of the six main anthocyanins present in red wine, B ring is respectively substituted by 1 (position 4′), 2 (positions 3′ and 4′) and 3 (3′, 4′ and 5′) –OH groups. According to its number of antioxidant –OH groups, pelagornidin has the lowest PAOT-Liquid® activity when compared to delphinidin and cyanidin as shown in Table 2.

Molecules from the flavanone family are characterized by the presence of one –OH group on ring A (position 5) and another one on ring C (position 4′). In the case of naringin and hesperidin methyl calcone, the –OH group on ring B (position 7) is substituted by a rutinose moiety, resulting in an important loss in the antioxidant capacity.

Table 3 shows that there was a clear shift between red, rosé and white wines with respect to their TPC. Mean TPC for red wines was 1789 ± 367 mg (GAE) L^{-1} against only 265 ± 65 for rosé and 221 ± 28 for white wines. These results are in agreement with literature data [18,19]. Among tested red wines, a large heterogeneity in TPC was evidenced. Two groups of values have been observed, those above (n = 5) or below (n = 9) 2000 mg (GAE) L^{-1}. However, we did not observe significant difference in TPC between red wines constituted of mono-, bi- or multi-varietals as also reported by Paixao et al. [19]. By contrast, a great homogeneity in low TPC was observed for rosé and white wines.

A shown in Figure 3, we evidenced that the PAOT-Liquid® activity and TPC of wines were highly correlated (r = 0.9540; $p < 0.0001$). Other authors using electrochemical detection with laccase biosensor [18], poly(3,4-ethylenedioxythiophene)-modified electrodes [20] or carbon nanotube-modified electrodes [21] reported similar findings. The high correlation between TPC and PAOT-Liquid® activity provided such strong evidence that the majority of the antioxidant activity was attributed to the polyphenolic compounds in such beverages. In a recent study [22], we concluded that the relative percentages of various classes of polyphenol compounds for red wines having only one grape variety (Merlot, Syrah, Cabernet Sauvignon) were as follows: 24.3% phenolic acids, 7.4% flavonols, 37.3% flavanols, 30.4% anthocyanidins and only 0.4% resveratrol (16). The grape variety Pinot Noir exhibited a different profile with less flavonols (2.8%) and anthocyanidins (14.6%), but more flavanols (54.9%).

Of interest was the evidence for a strong correlation between PAOT-Liquid® activity and DPPH assay as shown in Figure 4. Even if the wine matrix is the same in both assays, we have chosen to express the results in two different antioxidant scales. Indeed, chemical and synthetic Trolox was conventionally used as reference antioxidant molecule in all papers referring to DPPH assay. By contrast, it was more logical to express antioxidant activity of wines evaluated by the PAOT-Liquid® Technology by comparing to a natural antioxidant present in wine as it is the case for gallic acid. A great advantage of the PAOT-Liquid® Technology is that there is no interaction between the color of wine and those developed during the reaction of DPPH with the samples. At least, correlation was found between TPC of wines and the classical DPPH assay (Figure 5), as expected [23].

5. Conclusions

In conclusion, we have developed the PAOT-Liquid® Technology that turns out to be a direct and useful tool for evaluating antioxidant capacity of red, rosé and white wines. When compared to classical DPPH or ORAC assays which require long and fastidious protocols [24], the determination of antioxidant capacity of wines evaluated by the PAOT-Liquid® Technology was achieved within 10 min and without requiring analytical systems such as spectrophotometers or plaque readers, rendering the method easily accessible to the winemaker himself. One of the great weakness of classical DPPH or ORAC assays for measuring antioxidant capacity is also the absence of standardized protocols. In the literature, there are substantial differences in sample preparation, selection of end-points and expression of results [24], so that comparison between the values reported by different laboratories is quite difficult [25]. Thanks to its simple and automatized protocol, PAOT-Liquid® Technology overcomes these problems being operator independent.

Due to the strong correlation between antioxidant activity determined by the PAOT-Liquid® Technology and TPC and using a calibration curve, winemakers could, therefore, be able to quickly monitor themselves if modifications in TPC content occur or not from grape harvest until wine bottling and storage. At least, another advantage of the PAOT-Liquid® Technology is its moderate cost (around 10 €) when compared to more expensive tests performed in specialized laboratory analysis. Of interest is to note that the PAOT-Liquid® Technology can also be used for determining antioxidant capacity of other types of non-alcoholic beverages, such as orange juices or plant extracts, as already described by us [26].

Author Contributions: P.J. was the initiator of the study and has contributed to the paper draft with the help of E.E.R. K.C. and T.J. determined the total polyphenol content (TPC) in tested wines as well as their antioxidant activity by using DPPH assay. M.S. was the designer of the PAOT-Liquid® Technology. With, K.M.-M.'s help, he performed the PAOT-Liquid® activity determination in all tested wines.

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diseases

MDPI

Article

Wine and Olive Oil Phenolic Compounds Interaction in Humans

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Abstract: Extra virgin olive oil (EVOO) and red wine (RW) are two basic elements that form part of the so-called Mediterranean diet. Both stand out because of their high phenolic compound content and their potential related health benefits. The present study is focused on the metabolic disposition of resveratrol (RESV), tyrosol (TYR), and hydroxytyrosol (HT) following the consumption of EVOO, RW, and a combination of both. In this study, 12 healthy volunteers consumed a single dose of 25 mL of EVOO, 150 mL of RW, and a combination of both in a crossover randomized clinical trial. Urinary recovery of RESV, TYR, and HT was analysed in urine samples collected over a 6-h period following the intake of each treatment. Higher HT levels were observed following EVOO compared to RW (3788 $\pm$ 1751 nmols and 2308 $\pm$ 847 nmols respectively). After the combination of EVOO and RW, the recovery of TYR and HT metabolites increased statistically compared to their separate consumption (4925 $\pm$ 1751 nmols of TYR and 6286 $\pm$ 3198 nmols of HT). EVOO triggered an increase in glucuronide conjugates, while RW intake raised sulfate metabolites. Marginal effects were observed in RESV increased bioavailability after the combination of RW with the fat matrix provided by EVOO.

Keywords: hydroxytyrosol; tyrosol; resveratrol; EVOO; olive oil; RW; red wine; Mediterranean diet

1. Introduction

Research has shown that the Mediterranean diet (MD) reduces the risk of overall mortality and mortality associated with cardiovascular diseases, cancer, Parkinson's, and Alzheimer's [1]. Extra virgin olive oil (EVOO) and red wine (RW) represent two of the richest sources of phenolic compounds from the MD. They are thought to be major contributors to the beneficial health effects attributed to the MD. The main phenolic compounds present in EVOO are hydroxytyrosol (HT) and tyrosol (TYR) in the form of their respective secoiridoids oleuropein and ligstroside [2–4]. The most well-known polyphenol present in RW is resveratrol (RESV), mainly as its glucoside piceid. Nevertheless, RW contains a wide range of phenolic compounds with biological activities such as gallic acid, syringic acid, hydroxytyrosol, luteolin, and quercitin, among others [5].

RESV (3,4′,5-Trihydroxystilbene) is a natural stilbene present in grape products in two different isomers: the *trans*-isomers (*t*-RESV) and the *cis*-isomers (*c*-RESV). The skin and seeds are the richest parts of the grape. During the RW making process, the skin and the seeds are macerated, facilitating the extraction of RESV. Additionally, alcohol formation during fermentation facilitates this extraction. RESV is well absorbed in the intestine, but its bioavailability is limited because it is rapidly

metabolized [6,7]. RESV is a biologically active molecule, which has shown great potential in vitro and pre-clinical studies. The latter is both a chemo preventive and cardio protective agent. In addition, it also offers protection against diabetes, inflammation, and neuro degeneration [8,9]. However, clinical studies are limited and discrepancies have been found in the pre-clinical data. These discrepancies can in part be attributed to disparate doses and poor in vivo bioavailability. Therefore, strategies to increase the bioavailability of RESV are receiving increased attention [10].

HT and TYR have been widely studied in EVOO: both as the main antioxidants and for their potential health benefits. HT is one of the most potent dietary antioxidants. TYR possesses a structure similar to HT, but lacks a hydroxyl group; this results in a lower antioxidant activity compared to HT [11,12]. The EUROLIVE clinical trial provided evidence that olive oil phenolic compounds decreased LDL oxidation, a hallmark in the development of atherosclerosis [13]. As a result, the European Food Safety Agency (EFSA) released a health claim regarding olive oil phenolic compounds. EFSA recommended the ingestion of 5 mg of HT on a daily basis [2]. Furthermore, HT possesses antioxidant and anti-inflammatory properties, which have been shown to inhibit pathological processes involved in cardiovascular and neurodegenerative diseases [14,15]. Moreover, a recent study conducted within the framework of the PREDIMED trial associated high urinary excretion of homovanillyl alcohol (HVALc), a stable metabolite of HT, which provides protection against total mortality and cardiovascular diseases [16].

RW is a source of TYR and to a lesser extent, a source of HT. Both are produced as secondary metabolites of tyrosine during wine fermentation. Despite the low concentrations of HT in RW, significant amounts have been observed after RW ingestion [17]. In this context, it is worthwhile to mention that there is an endogenous formation of HT following ethanol administration. HT is normally produced as a minor metabolite of dopamine oxidative metabolism (also known as DOPET). However, after ethanol administration, dopamine oxidative metabolism is shifted to metabolic pathways, resulting in a significantly higher production of HT in a dose-dependent manner [18]. Nevertheless, the higher recovery of HT after RW consumption could not be explained simply by considering the ethanol-induced formation. Further pre-clinical studies identified TYR as the metabolic precursor of HT [19]. TYR is endogenously bio transformed in humans into HT by means of the isoforms CYP2A6 and CYP2D6 [20]. HT endogenous generation after RW consumption could, in part, explain the beneficial effects derived from moderate wine consumption [17]. Nevertheless, it is worth mentioning that according to the EFSA, a diet containing more than 1.2% of alcohol by volume could not bear any health claims [21].

In the context of the Mediterranean diet, the respective effects of EVOO and RW have been studied extensively. However, to our knowledge, no study investigating the interaction between the metabolic dispositions of their phenolic compounds has yet been conducted. A typical Mediterranean meal includes a serving of EVOO as the fatty component, and a glass of RW. Consumed at the same time, EVOO and RW could interact synergistically, potentiating the bioavailability of their phenolic compounds. The latter would then benefit from the interaction between the hydro-alcoholic properties of RW and the fatty matrix of EVOO, and finally have an impact attenuating the postprandial associated oxidative stress and hyperlipidemia. The aim of the present study was to evaluate RESV, TYR, and HT metabolic disposition after the consumption of EVOO and RW, as well as to assess the potential synergy of its combination on the bioavailability of their phenolic compounds.

2. Materials and Methods

2.1. Subjects and Study Design

The study consisted of a crossover randomized clinical trial with three different interventions. The interventions consisted of a single administration of 25 mL of EVOO, 150 mL of RW, or the combination of both 25 mL of EVOO and 150 mL of RW. Treatment quantities were equivalent to normal dietary doses of a typical MD. A total of twelve healthy subjects (50% women, 34.0 $\pm$ 10.5,

BMI = 22.0 ± 3.3 kg/m^2) participated in the study. The study was explained to participants through verbal and written instructions, and written informed consent was obtained before participation. Volunteers received each treatment in a randomized manner. The study included three experimental sessions in which each intervention was administered under fasting conditions. Each experimental session was preceded by a two-day washout period. The total duration of the study was nine days.

To standardize baseline concentrations of phenolic compounds, subjects were asked to follow a low phenolic content diet, in which participants excluded olive oil and derivates, grapes and derivatives, and all alcoholic beverages from their diet. The low phenolic content diet was followed for two days prior to each intervention and during each experimental session.

On the day of the intervention, subjects consumed one of the three interventions within a period of 5–10 min. Urine was collected in separate fractions, at baseline (−2–0 h), to assess dietary compliance and during 6 h after each dietary intervention (0–6 h). The amount of urine in each fraction was measured, acidified with 6 M HCl, and stored at −20 °C until analysis. The study protocol was approved by the Ethics Committee of Parc de Salut Mar (CEIC-PSMAR) (Spain), and the clinical trial was registered at the International Standard Randomized Controlled Trial Number (NCT03614520).

2.2. Red Wine and Extra Virgin Olive Oil

Red wine of the Merlot variety (Cristiari d'Alòs 2014, 13% *v*/*v* ethanol, Costers del Segre, Lleida, Spain) was selected for its high content of resveratrol and piceid (reservatrol-3-β-mono-D-glucoside) in comparison to other red wine varieties [22]. EVOO administrated in the study was produced from arbequina olives obtained directly from olives and extracted solely by mechanical means (Germanor, Les Borges Blanques, Lleida, Spain).

2.3. Standards and Reagents

Diethylstilbestrol (internal standard (IS)), β-glucuronidase from *Helix pomatia* type H-2, homovanillyl alcohol (HVALc), 3-(4-hydroxyphenyl)-1-propanol, HT, piceid, *t*-RESV, and Tyr were purchased from Sigma-Aldrich (St Louis, MO, USA). Ethyl glucuronide, HVALc-glucuronide, HT-acetate-sulfate, HT-glucuronide, HT-3-sulfate, TYR-glucuronide, and TYR-sulfate, as well as the internal standards ethyl-glucuronide-d_5, 4-(3-hydroxypropyl) phenyl glucuronide, HT-D_3, and HT-1-sulfate, were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). Ammonium iodide (NH_4I), formic acid (H-COOH), hydrochloric acid (HCl), 2-mercaptoethanol, phosphoric acid, sodium acetate, sodium chloride (NaCl), sodium hydroxide (NaOH), and sodium metabisulfite were purchased from Merck (Darmstadt, Germany). Acetonitrile, ethyl acetate, and methanol were supplied by Scharlab SL (Barcelona, Spain), while *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was supplied by Macherey–Nagel (Düren, Germany). Dihydroresveratrol and *c*-RESV were prepared from *t*-RESV as previously reported [3]. Oasis HLB 3cc Vac Cartridges (60 mg) (WAT094226) for solid-phase extraction were purchased from Waters Corporation (Milford, MA, USA). Ultrapure water (Milli-Q) was obtained from a Millipore system (Millipore, Bedford, MA, USA) and blank human urine from volunteers after three days of a diet restricted in alcohol, grape, and olive derivates.

2.4. Extraction and Analysis of Resveratrol in Red Wine and Urine Samples

2.4.1. Red Wine

RESV and piceid content in RW was measured by gas chromatography coupled to mass spectrometry (GC-MS) after a liquid-liquid extraction, as previously described [6]. In short, 1 mL of diluted red wine (1:10 in water) was extracted with 5 mL ethyl acetate in amber glass tubes (to avoid *t*-RESV conversion to its *cis* form). After being shaken for 30 min, samples were centrifuged for 5 min at 300 g and the supernatant was transferred to another amber tube to be evaporated until dry by a sample concentrator (Caliper Life Sciences, Waltham, Massachusetts, MA, USA) at 30 °C under a stream of nitrogen. After 1 h in an oven at 50 °C, the residue was derivatized with 75 µL of

MSTFA: NH4I:2-mercaptoethanol reaction mixture (2 g NH_4I and 5 mL of 2-mercaptoethanol per liter of MSTFA) for 30 min at 60 °C. Calibration curves were prepared by adding different concentrations of *c*- and *t*-RESV and piceid (100–1000 μg/L) to water (10 mL) and extracted in the same way as red wine samples. Finally, 2 μL was injected into the gas chromatograph.

2.4.2. Urine Samples

Urine was subjected to a hydrolysis procedure previously described by our working group [3]. Aliquots of 1 mL of diluted urine (1:10 in water) were spiked with 10 μL of IS (containing 10 μg/mL of diethylstilbestrol), 100 μL of sodium metabisulfite, 1 mL acetate buffer 0.1 M pH 5.2, and 25 μL of β-glucuronidase. The samples were incubated at 37 °C overnight. After incubation, 1 mL of NaOH was added to neutralize the hydrolysis process. For the extraction of the phenolic compounds, 0.5 mL of a saturated solution of NaCl and 4 mL of acetonitrile-ethyl acetate mixture (1:4 *v*/*v*) were added. Samples were mixed for 30 min, centrifuged for 5 min at 300 g, and the organic phase was extracted and evaporated until dry. After 1 h in an oven at 50 °C, the residue was derivatized with 75 μL of MSTFA: NH4I:2-mercaptoethanol reaction mixture for 30 min at 60 °C and 2 μL was injected into the gas chromatograph.

For the preparation of the calibration curves, 1 mL of diluted urine (1:10 in water) was spiked with an increasing concentration of *c*- and *t*-RESV and dihydro-RESV (10–200 ng/mL), and subjected to the extraction procedure exactly in the same way as the samples.

The extracted samples were analyzed using an Agilent Technologies (Santa Clara, California, CA, USA) 6890 N gas chromatograph coupled to a 5973 mass-selective detector. For chromatographic separation, a 5% phenyl-dimethyl-polysiloxane Zebron™ (Torrance, CA, USA) fused-silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) was used. The split injection mode using helium as a carrier gas (0.9 mL/min) was applied. The temperatures of the injector and transfer line were set at 280 °C. Gas chromatographic conditions were as follows: initial oven temperature at 80 °C, raised by 20 °C/min to 200 °C, then by 10 °C/min to 300 °C, and maintained at 300 °C for 3 min. The mass spectrometer was operated in the selected ion monitoring mode (SIM) and had an electron impact of 70 eV. Ions at *m*/*z* 444 (RESV and piceid), *m*/*z* 179 (dihydro-RESV), and *m*/*z* 412 (diethylstilbestrol), were selected for the quantitative analysis. For the confirmation of the compounds, the ions chosen were *m*/*z* 445 for RESV and piceid, *m*/*z* for 446 dihydro-RESV, and *m*/*z* 397 and 383 for diethylstilbestrol.

2.5. Extraction and Analysis of Hydroxytyrosol in Extra Virgin Olive Oil, Red Wine and Urine Samples

2.5.1. Extra Virgin Olive Oil

TYR and HT content in EVOO were quantified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) after a triple liquid-liquid extraction, as previously described [23]. Briefly, aliquots of 1 mL of olive oil were spiked with 10 μL of IS (containing 100 μg/mL of HT-D_3 and 3-(4-hydroxyphenyl)-1-propanol). A first liquid-liquid extraction was performed with 10 mL of methanol/water solution (80:20, *v*/*v*) containing 1 mM of ascorbic acid to avoid phenol degradation during the process. Tubes were shaken for 60 min, and then centrifuged (2000 g, 5 min). The organic phase was transferred into a new tube and evaporated under a nitrogen stream at 30 °C to a final remaining volume of 2 mL of an aqueous extract of olive oil. Thereafter, metabisulfite was added to the samples to prevent oxidation. To hydrolyze all the conjugated forms of TYR and HT, samples were incubated at 37 °C for 30 min with HCl (1.5 mmol/tube), to mimic gastrointestinal conditions during digestion. Following that, a liquid-liquid extraction was performed by adding 4 mL of a mixture of ethyl acetate and acetonitrile (4:1 *v*/*v*) shaking for 30 min and centrifuging (2000 g, 5 min). The organic phase was transferred into a new tube and the liquid-liquid extraction was repeated, finally combining both organic phases into the same tube and evaporating the mixture until completely dry. Extracts were reconstituted with 100 μL of mobile phase containing (80% A: 20% B) and injected into the

LC-MS/MS. The composition of mobile phase A was 0.01% of ammonium acetate (pH 5) in water; mobile phase B was pure methanol. Calibration curves were prepared by adding standards of TYR and HT to 1 mL of refined oil. All the samples were analyzed in triplicate.

Samples were analyzed using an Agilent Technologies 6410 Triple Quad (Santa Clara, CA, USA). The separation was carried out with an Acquity UPLC® BEH C18 column (Waters, Milford, MA, USA) with a 1.7 μm particle size, 3 mm × 100 mm (Waters, Milford, MA, USA). The injection volume was 10 μL and the ion source operated in negative ionization mode.

2.5.2. Red Wine

Red wine content of TYR and HT was determined by LC-MS/MS after a simple dilution. Briefly, red wine samples were diluted 40 times with mobile phase (65% A: 35% B) spiked with 10 μL of IS (containing 10 μg/mL of hydroxytyrosol-D_3 and 3-(4-hydroxyphenyl)-1-propanol). Calibration curves were prepared by adding standards of TYR and HT to pure water. All samples were analyzed in triplicate. The composition of mobile phase A was 0.01% of ammonium acetate (pH 5) in water; mobile phase B was pure methanol. Samples were analyzed using an Agilent Technologies 6410 Triple Quad (Santa Clara, CA, USA). The separation was carried out with an Acquity UPLC® BEH C18 column 1.7 μm particle size, 3 mm × 100 mm (Waters, Milford, MA, USA). The injection volume was 10 μL and the ion source operated in negative ionization mode.

2.5.3. Urine Samples

The quantification of urinary levels of HT and TYR free forms and their metabolites was performed using a solid-phase extraction and following the method previously described [24,25]. The method was capable of detecting the free forms HT, TYR, and HVALc; the sulfate conjugates HT-sulfate, TYR-sulfate, and HT-acetate-sulfate; and the glucuronide conjugates HT-glucuronide, TYR-glucuronide, and HVALc-glucuronide. Shortly thereafter, aliquots of 0.5 mL of the samples were diluted with 0.5 mL of purified water and spiked with 10 μL of internal standard (containing 10 μg/mL of HT-D_3, 3-(4-hydroxyphenyl)-1-propanol, 4-(3-hydroxypropylphenyl) glucuronide and HT-1-sulfate) and stabilized with 1 mL of phosphoric acid 4%. Thereafter, samples were submitted to a solid-phase extraction by means of Oasis HLB columns. Samples were loaded into the cartridges, and the cartridges were then washed with 2 mL of purified water. Finally, the compounds of interest were eluted by adding 2 mL of methanol to the cartridges. Subsequently, the methanol was evaporated until dry using nitrogen (29 °C, 10–15 psi). Finally, the dried extracts were reconstituted in 100 μL of a mixture of mobile phases (91% A/9% B v/v), transferred into HPLC vials, and analyzed by LC-MS/MS.

Identification and quantification of HT and TYR metabolites was performed using an Agilent 1200 series HPLC system (Agilent technologies, Santa Clara, CA, USA) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface. The chromatographic separation was carried out with an Acquity UPLC® BEH C18 column with a 1.7 μm particle size, 3 mm × 100 mm (Waters, Milford, MA, USA) maintained at 40 °C. The composition of mobile phase A was 0.01% of ammonium acetate (pH 5) in water; mobile phase B was pure methanol. The injection volume was 10 μL and the ion source operated in negative ionization mode.

2.6. Ethyl Glucuronide Quantification

Ethyl glucuronide concentration in urine was used as a marker of alcohol abstinence and compliance with the dietary recommendations before each intervention. To determine urinary concentrations of ethyl glucuronide, aliquots of 30 μL were mixed with 10 μL of IS mix solution (containing 10 μg/mL ethyl-glucuronide-d_5 and ethyl-sulfate-d_5) and 110 μL of 0.1% formic acid solution in water. The identification and the quantification of ethyl glucuronide were carried out using an Agilent 1200 series HPLC system (Agilent technologies) (Santa Clara, California, CA, USA) coupled to a triple quadrupole (6410 Triple Quad LC/MS; Agilent) mass spectrometer with an electrospray interface. To perform the chromatographic separation, an Acquity UPLC® BEH C18 column with

a 1.7 μm particle size, 3 mm × 100 mm (Waters, Milford, MA, USA) was used. The composition of mobile phase A was 0.1% (*v*/*v*) formic acid in water, and mobile phase B was 0.1% (*v*/*v*) formic acid in acetonitrile.

2.7. Statistical Analysis

Primary outcomes were HT, TYR, and RESV urinary recovery. Sample size calculation was based on HT urinary recovery and indicated that a total of 12 volunteers was enough to detect a difference of 1000 nmol of HT with a power of 90% and $\alpha = 0.05$. All the results were subjected to a normality test prior to the statistical analysis and then to one-way analysis of variance (ANOVA) with the Bonferroni post hoc test in the case of homogeneity of variances and T3 Dunnett when the variances were not homogenous. The results were reported as the mean ± standard deviation (SD). Differences at $p < 0.05$ were considered statistically significant. SPSS software (Version 18.0, Japan Inc., Tokyo, Japan) was used for data analysis.

3. Results

3.1. Phenolic Content in Extra Virgin Olive Oil and Red Wine

In order to characterize the principal phenolic compounds of the interventions, EVOO and RW were analyzed in triplicate. Table 1 shows the concentration observed for RESV and its isomers, corresponding to RW, and the concentration of HT and TYR of RW and EVOO treatments.

RW contained a concentration of 2.4 ± 0.1 mg/L of *t*-RESV, while the concentration of *c*-RESV was found to be 3.0 ± 0.4 mg/L. Regarding piceid, *t*-piceid was determined at a concentration of 4.9 ± 0.2 mg/L and its isomer *cis* obtained a concentration of 3.0 ± 0.5 mg/L. HT and TYR were also analyzed in RW with concentrations of 1.5 ± 0.1 and 35.0 ± 1.0 mg/L, respectively, and in EVOO, HT was detected at a concentration of 19.8 ± 1.9 mg/L and TYR at 24.1 ± 2.8 mg/L.

Therefore, for RW treatments (150 mL), about 0.36 mg of *t*-RESV, 0.45 mg of *c*-RESV, 0.74 mg of *t*-piceid, 0.45 mg of *c*-piceid, 0.22 mg of HT, and 5.25 mg of TYR were administered. Regarding EVOO, 25 mL of the dose administrated corresponds to 0.50 mg of HT and 0.60 mg of TYR.

Table 1. Phenolic content of EVOO, RW, and administered doses.

Treatment	Concentration (mg/L)						Dose Administered (mL)	Dose Administered (mg)					
	t-RESV	*c*-RESV	*t*-Piceid	*c*-Piceid	HT	TYR		*t*-RESV	*c*-RESV	*t*-Piceid	*c*-Piceid	HT	TYR
EVOO	0	0	0	0	19.8 ± 1.9	24.1 ± 2.8	25	0	0	0	0	0.50	0.60
RW	2.4 ± 0.1	3.0 ± 0.4	4.9 ± 0.2	3.0 ± 0.4	1.5 ± 0.1	35.0 ± 1.0	150	0.36	0.45	0.74	0.45	0.22	5.25
EVOO + RW	NA	NA	NA	NA	NA	NA	25 + 150	0.36	0.45	0.74	0.45	0.72	5.85

Phenolic composition of EVOO and RW and the equivalent doses administered in the study. Data expressed as mean ± SD.

3.2. Quantification of Phenolic Compounds in Urine

3.2.1. Baseline

Diet compliance was assessed by the baseline analysis of urine samples collected 2 h before the beginning of the intervention (−2 to 0 h). Volunteers followed the washout recommendations perfectly since no RESV was observed in baseline urine samples. In terms of HT, TYR, and metabolites, as they are endogenous compounds, traces could be observed in baseline samples, but these were not attributed to the diet contribution.

3.2.2. Resveratrol

Urinary amounts of RESV and its isomers were analyzed in the three interventions, both at baseline and 6 h after consumption. Table 2 shows the concentration of the phenolic compounds found during the study. Only when RW was administered, RESV and its isomers were identified (Figure 1). Urinary recovery of *t*-RESV after 6 h in the RW treatment was 59.2 ± 28.7 nmol, while after RW + EVOO, the concentration of this compound reached 61.7 ± 42.4 nmol. Similar results were obtained with dihydro-RESV, which increased its concentration following RW + EVOO treatment compared with RW (13.0 ± 8.3 nmol RW + EVOO vs. 10.9 ± 7.5 nmol RW). Regarding *c*-RESV, the compound also presented a greater increase in RW + EVOO treatment, although none of the interventions presented significant differences, probably due to high interindividual differences.

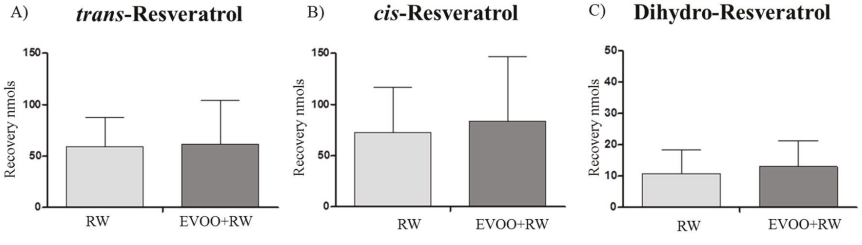

Figure 1. RESV urinary recovery (nmol) from 0 to 6 h after RW and RW + EVOO of (**A**) *t*-RESV; (**B**) *c*-RESV; and (**C**) Dihydro-RESV. Data expressed as mean ± SD.

Table 2. RESV urinary recovery (nmol) from 0 to 6 h after treatments.

Phenolic Compound (nmols)	EVOO	RW	EVOO + RW
t-RESV	0.0 ± 0.0	59.2 ± 28.7 ^{aa}	61.7 ± 42.4 ^{aa}
c-RESV	0.0 ± 0.0	72.8 ± 44.3 ^{aa}	83.8 ± 62.6 ^{aa}
Dihydro-RESV	0.0 ± 0.0	10.9 ± 7.5 ^{aa}	13.0 ± 8.3 ^{aa}

Urinary excretion 0–6 h of *t*-RESV, *c*-RESV, and dihydro-RESV after EVOO, RW, and EVOO + RW (n = 12). Data expressed as mean ± SD. ^{aa} $p < 0.01$ versus EVOO.

3.2.3. Hydroxytyrosol

HT and its metabolites were analyzed by LC-MS/MS, obtaining a total of nine metabolites found in urine after the three interventions (Table 3). The analytical method included the quantification of the free forms and phase II metabolites conjugated with sulfate and glucuronide. Figure 2 shows the sum of HT metabolites (Figure 2A) and TYR metabolites (Figure 2B) after each intervention. Figure 2A shows differences in HT recovery between the three interventions. EVOO + RW had the highest recovery. Similar results were obtained regarding TYR metabolites, and significant differences were obtained between EVOO + RW and RW and between EVOO + RW and EVOO, but no difference was observed in TYR recovery between EVOO and RW.

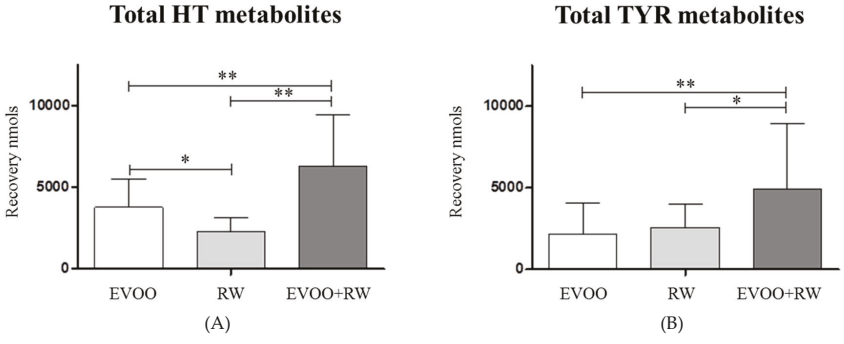

Figure 2. HT and TYR urinary recovery (nmol) from 0 to 6 h after EVOO, RW, and RW+EVOO (n = 12) of (**A**) Total HT (HT-glucuronide + HT-sulfate + HT-acetate-sulfate + free HT + HVALc free + HVALc glucuronide) and (**B**) Total TYR (Tyrosol-glucuronide + TYR-sulfate + free Tyrosol). Data expressed as mean ± SD. * $p < 0.05$; ** $p < 0.01$.

Table 3. HT, TYR, and metabolites urinary recovery (nmol) from 0 to 6 h after treatments.

Phenolic Compound (nmols)	EVOO	RW	EVOO + RW
Total HT	3788 ±1751	2308 ± 847 ^{a}	6286 ± 3198 $^{aa\ bb}$
Total TYR	2180 ± 1917	2567 ± 1468	4925 ± 3993 $^{aa\ b}$
Free HT	367 ± 221	201 ± 173 ^{aa}	386 ± 289 ^{bb}
Free TYR	404 ± 346	132 ± 114 ^{aa}	460 ± 490 ^{b}
Free HVALc	269 ± 145	110 ± 118 ^{aa}	247 ± 205 ^{bb}
HT-sulfate	1336 ± 795	1767 ± 787	3655 ± 1926 $^{aa\ b}$
TYR-sulfate	138 ±194	1133 ± 1052 ^{aa}	1252 ± 1190 ^{bb}
HT-acetate-sulfate	465 ± 528	11.2 ± 30.9 ^{aa}	436 ± 543 ^{bb}
HT-glucuronide	974 ± 766	90.5 ± 56.3 ^{aa}	1000 ± 856 ^{bb}
TYR-glucuronide	1639 ± 1438	1301 ± 720	3215 ± 2421 $^{aa\ b}$
HVALc-glucuronide	376 ± 284	139 ± 114 ^{a}	563 ± 401 ^{bb}

Urinary excretion 0–6 h of total HT metabolites, total TYR metabolites, and single metabolites after EVOO, RW, and EVOO + RW (n = 12). Data expressed as mean ± SD. ^{a} $p < 0.05$, ^{aa} $p < 0.01$ versus EVOO; ^{b} $p < 0.05$, ^{bb} $p < 0.01$ versus RW. Total HT = HT-glucuronide + HT-sulfate + HT-acetate-sulfate + free HT + HVALc free + HVALc glucuronide); Total Tyrosol = Tyrosol-glucuronide + TYR-sulfate + free Tyrosol.

When analyzing the different metabolic pathways in more depth after each intervention, it was observable that free forms were present at low concentrations (between 10–15% of the total), while the sulfate and glucuronide conjugates were the most abundant. HT-sulfate increased following both interventions, whereas TYR-sulfate was increased exclusively after RW; on the contrary, HT-sulfate-acetate was only present after EVOO. When comparing glucuronides, HT-glucuronide was only generated after the EVOO treatment, and HVALc-glucuronide was detectable after RW, but its major contributor was EVOO. Finally, TYR-glucuronide increased equally after both interventions (Figure 3).

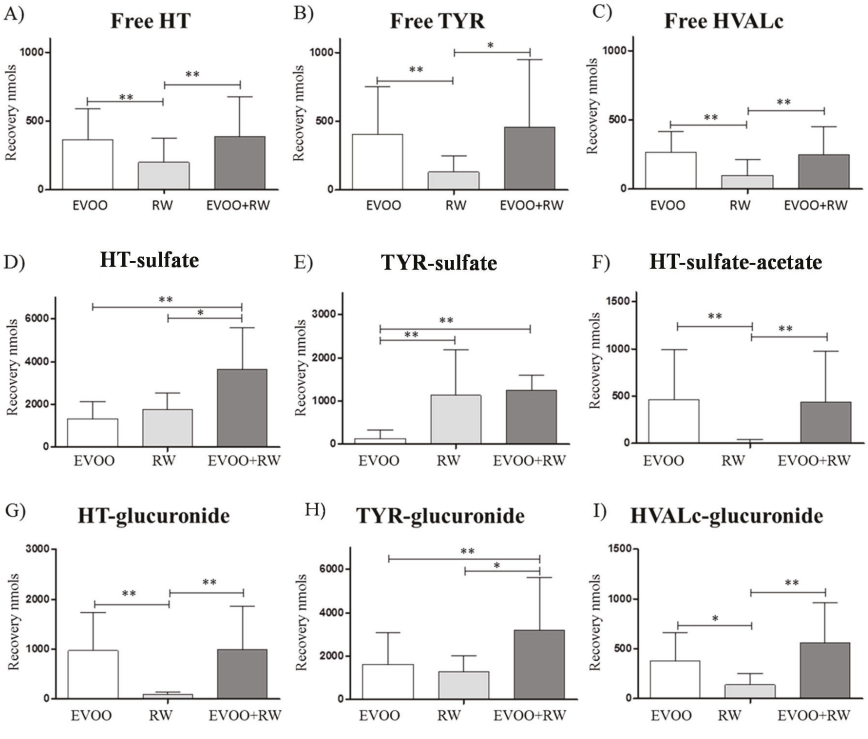

Figure 3. HT and TYR free forms, sulfate, and glucuronide metabolites urinary recovery (nmol) from 0 to 6 h after EVOO, RW, and RW + EVOO (n = 12) of (**A**) Free HT; (**B**) Free TYR; (**C**) Free HVALc; (**D**) HT-sulfate; (**E**) TYR-sulfate; (**F**) HT-sulfate-acetate; (**G**) HT-glucuronide; (**H**) TYR-glucuronide; and (**I**) HVALc-glucuronide. Data expressed as mean ± SD. * $p < 0.05$; ** $p < 0.01$.

3.3. Ethyl Glucuronide

Ethyl glucuronide at baseline was undetectable, confirming that volunteers followed an alcohol-free diet. Ethyl glucuronide recovery was almost identical after RW intervention (19.0 ± 9.5 μmols) and RW + EVOO intervention (19.3 ± 7.6 μmols), while it was undetectable after EVOO.

4. Discussion

It is the first time in humans that the interaction between RW and EVOO is evaluated in terms of the metabolic disposition of the main phenolic compounds of both Mediterranean food components. Here, we report that recoveries of phenolic compounds from RW and EVOO are altered when combining both foods. There is a recovery of HT and related compounds that doubles the expected concentrations taking into consideration amounts of HT present in RW and EVOO at the doses administered. On the other hand, a non-significant increase of resveratrol-related compounds is observed when combining RW and EVOO.

The study of the interaction of both foods is of relevance since phenolic compounds typically have a poor bioavailability, being very much dependent on the matrix in which they are present [6,10,26]. The mixture of a fatty matrix and a hydro-alcoholic one may influence its bioavailability. Some preliminary results suggest that this would be the case and that the interaction may result in beneficial health effects [27].

In the case of HT, we previously reported that there is an interaction between alcohol and phenolic components of RW, in particular, TYR. Alcohol, on the one hand, interacts with dopamine oxidative metabolism, promoting a shift in its metabolic pathways. A minor pathway from DOPAL (3,4-Dihydroxyphenylacetaldehyde) to DOPET (3,4-dihydroxyphenylethanol, also known as HT) becomes more apparent in the presence of alcohol. In humans, we have demonstrated that DOPET (HT) generation is alcohol dose dependent [18]. Similarly there is an increased synthesis of TYR via tyrosine metabolic disposition in an analogous way, as described for the dopamine and ethanol interaction. Although there is a contribution of dopamine and tyrosine when alcohol is consumed in the formation of HT and TYR, this does not suffice to explain recoveries of HT and TYR [19]. We demonstrated in vivo, in animal models, and in vitro, in human liver biopsies, that the most likely explanation for higher recoveries of both phenolic compounds is the biotransformation of TYR to HT, a reaction regulated by the polymorphic enzymes CYP2D6 and CYP2A6 [20]. Ethanol contributes to this reaction by increasing TYR bioavailability and then favoring the biotransformation reaction. Here, we demonstrate for the first time that the contribution of this reaction leading to HT formation by RW is quite substantial when compared to recoveries after EVOO. The dose of total HT contained in RW represents 44% of the one contained in EVOO. Nevertheless, the total HT recovery following RW represents 60% of the recovery after EVOO. This observation confirms an endogenous formation of HT following RW consumption.

The metabolic disposition of HT has been reviewed recently [2]. When comparing the metabolic pathways of HT for EVOO and RW, a higher recovery of unaltered HT is observed after EVOO. Therefore the impact of HT from EVOO when compared to RW in terms of biological effects would be superior. Nonetheless, when looking at metabolic pathways, it is apparent that HT-sulfate and TYR-sulfate recoveries are higher after RW than after EVOO, and 60% of the total metabolites were sulfate conjugates in the case of RW compared with the 32% in the case of EVOO. This is of relevance since these metabolites have been reported to be biologically active, specifically preventing the effects of oxidized cholesterol, and not the corresponding glucuronide metabolite [28].

Regarding resveratrol, marginally higher concentrations of *t*-RESV, *c*-RESV, and dihydro-RESV are observed after RW combined with EVOO, suggesting that resveratrol bioavailability is slightly increased in the presence of EVOO.

Author Contributions: A.B., M.M.-H., and A.C. analyzed the samples. A.B. and M.M.-H. wrote the manuscript. R.d.l.T. conceived the experiment, and wrote and critically revised the manuscript.

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

Abbreviations

NH_4I	ammonium iodide;
EFSA	European Food Safety Agency;
EVOO	extra virgin olive oil;
GC-MS	gas chromatography coupled to mass spectrometry;
H-COOH	formic acid;
HVALc	homovanillyl alcohol;
HCl	hydrochloric acid;
HT	hydroxytyrosol;
IS	internal standard;
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry;
MD	Mediterranean diet;
MSTFA	*N*-methyl-*N*-trimethylsilyltrifluoroacetamide;
RW	red wine;

RESV	resveratrol;
NaCl	sodium chloride;
NaOH	sodium hydroxide;
TYR	tyrosol.

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Induction of Neuronal Differentiation of Murine N2a Cells by Two Polyphenols Present in the Mediterranean Diet Mimicking Neurotrophins Activities: Resveratrol and Apigenin

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Abstract: In the prevention of neurodegeneration associated with aging and neurodegenerative diseases (Alzheimer's disease, Parkinson's disease), neuronal differentiation is of interest. In this context, neurotrophic factors are a family of peptides capable of promoting the growth, survival, and/or differentiation of both developing and immature neurons. In contrast to these peptidyl compounds, polyphenols are not degraded in the intestinal tract and are able to cross the blood–brain barrier. Consequently, they could potentially be used as therapeutic agents in neurodegenerative pathologies associated with neuronal loss, thus requiring the stimulation of neurogenesis. We therefore studied the ability to induce neuronal differentiation of two major polyphenols present in the Mediterranean diet: resveratrol (RSV), a major compound found in grapes and red wine, and apigenin (API), present in parsley, rosemary, olive oil, and honey. The effects of these compounds (RSV and API: 6.25–50 μM) were studied on murine neuro-2a (N2a) cells after 48 h of treatment without or with 10% fetal bovine serum (FBS). Retinoic acid (RA: 6.25–50 μM) was used as positive control. Neuronal differentiation was morphologically evaluated through the presence of dendrites and axons. Cell growth was determined by cell counting and cell viability by staining with fluorescein diacetate (FDA). Neuronal differentiation was more efficient in the absence of serum than with 10% FBS or 10% delipidized FBS. At concentrations inducing neuronal differentiation, no or slight cytotoxicity was observed with RSV and API, whereas RA was cytotoxic. Without FBS, RSV and API, as well as RA, trigger the neuronal differentiation of N2a cells via signaling pathways simultaneously involving protein kinase A (PKA)/phospholipase C (PLC)/protein kinase C (PKC) and MEK/ERK. With 10% FBS, RSV and RA induce neuronal differentiation via PLC/PKC and PKA/PLC/PKC, respectively. With 10% FBS, PKA and PLC/PKC as well as MEK/ERK signaling pathways were not activated in API-induced neuronal differentiation. In addition, the differentiating effects of RSV and API were not inhibited by cyclo[DLeu^5] OP, an antagonist of octadecaneuropeptide (ODN) which is a neurotrophic factor. Moreover, RSV and API do not stimulate the expression

of the diazepam-binding inhibitor (DBI), the precursor of ODN. Thus, RSV and API are able to induce neuronal differentiation, ODN and its receptor are not involved in this process, and the activation of the (PLC/PKC) signaling pathway is required, except with apigenin in the presence of 10% FBS. These data show that RSV and API are able to induce neuronal differentiation and therefore mimic neurotrophin activity. Thus, RSV and API could be of interest in regenerative medicine to favor neurogenesis.

Keywords: N2a murine neuronal cells; neuronal differentiation; neurotrophic effects; polyphenols; apigenin; resveratrol

1. Introduction

Polyphenols belong to one of the most abundant phytochemicals in the plant kingdom. They are the result of the secondary metabolism of plants through two fundamental metabolic pathways: the shikimate pathway and the acetate pathway [1,2]. There are currently about 8000 different polyphenols, divided into at least 10 different classes based on their chemical structure. They are classified as: (1) flavonoids including flavanols, isoflavones, flavanones, flavonones, and anthocyanidins; and (2) nonflavonoids such as phenolic acids (groups of compounds derived from benzoic and hydroxycinnamic acids), stilbenes, and lignans; tannins are flavonoid polymers [3,4].

Polyphenols, which have nutritional interest as micronutrients, are particularly abundant in several foods (vegetables, fruits), oils (argan and olive oils) and beverages (red wines) associated with in the Mediterranean diet. There is much evidence from in vitro studies, animal models, and clinical studies supporting that polyphenol compounds may have geroprotective activities as well as cytoprotective effects, especially in age-related diseases (cardiovascular diseases, eye diseases (cataracts, age-related macular degeneration) and chronic diseases (bowel diseases)) associated or not with enhanced oxysterol levels [5,6], through the control of mitochondrial dysfunctions, oxidative stress, inflammation, angiogenesis, and cell death [7,8]. At the brain level, phytosterols could prevent oxytosis, i.e., oxidative stress-induced cell death, which could play major role in neurodegeneration [9,10]. There is also lot of evidence that several polyphenols have anti-tumor properties (cell cycle delay, apoptosis induction, metastasis prevention) [11,12]. Interestingly, there is now recent evidence that polyphenols (especially resveratrol, a polyphenol of the stilbene family, found in grapes, blackberries, or peanuts for example) have pro-differentiating properties on different cell types: adipocytes, hematopoietic cells, human umbilical cord mesenchymal stem cells, cancer cells (thyroid, glioblastoma, colon), human lung fibroblasts, keratinocytes, embryonic cardiomyoblasts, and myoblasts [13–18]. There is also now evidence that many dietary components of the Mediterranean diet (curcumin, resveratrol, and polyunsaturated fatty acids (PUFAs)), and diets enriched with polyphenols and PUFAs, as well as caloric restriction, physical exercise, and learning, are able to induce neurogenesis in the adult brain. It is therefore tempting to speculate that nutritional approaches, functional foods enriched in polyphenols, or functionalized polyphenols (polyphenols coupled with nanoparticles) [19,20] could provide promising prospects to stimulate adult neurogenesis and combat neurodegenerative diseases and cognitive decline [21,22].

In addition, several polyphenols, including flavonoids such as baicalein, daidzein, luteolin, and nobiletin as well as non-flavonoid polyphenols such as auraptene, carnosic acid, curcuminoids, and hydroxycinnamic acid derivatives including caffeic acid phentyl ester have neurotrophic effects: they enhance neuronal survival and promote neurite outgrowth in vitro, a hallmark of neuronal differentiation [23]. Flavonoids are also able to induce neuronal differentiation of mouse embryonic stem cells and human pluripotent stem cells [24]. Altogether, these data support the neurotrophic effects of polyphenols. They also support the ability of these molecules to mimic the functions and/or to stimulate the production of neurotrophic factors, which are a family of biomolecules (peptides

or small proteins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and octadecaneuropeptide (ODN)) capable of favoring the growth, survival, and/or differentiation of both developing and mature neurons [23,25,26]. This is in contrast to peptidyl compounds such as neurotrophins; since polyphenols are not degraded in the intestinal tract and are able to cross the blood–brain barrier [26,27], they could potentially be used as therapeutic agents in neurodegenerative pathologies associated with neuronal loss such as ischemic stroke, Alzheimer's and Parkinson's diseases, which require the stimulation of neurogenesis [23,28,29]. At the time, while it is considered that polyphenols can mimic neuroprotective activities, little is still known about the ability of these molecules to favor neuronal differentiation and the associated metabolic pathways. Resveratrol, which is an important component of the Mediterranean diet, has been reported to have antioxidant and antitumor properties, but its effects as a neural plasticity inducer are still debated [30]. Apigenin (a chemical compound of the family of flavones, a subclass of flavonoids) is a major polyphenol of parsley, which is also much consumed in the Mediterranean diet, mainly in the Middle East where it is also used in traditional and folklore medicines [31]. Apigenin is found in thyme, rosemary, celery and chamomile; it is also present in honey [32] as well as in olive oil [33]. At the moment, apigenin has been shown to modulate GABAergic and glutamatergic transmission in cultured cortical neurons [34]. Neuroprotective, anti-amyloidogenic, and neurotrophic effects of apigenin have been reported in an Alzheimer's disease mouse model (APP/PS1) [17]. These effects were associated with an activation of cyclic adenosine monophosphate response element-binding protein (CREB), characterized by an increased level of phosphorylated CREB [17].

In the present study, based on the ability of polyphenols to mimic the action of neurotrophic compounds (cytoprotection and/or differentiation), we asked whether two major polyphenols present in the Mediterranean diet (trans-resveratrol (RSV) and apigenin (API)) were able to induce neuronal differentiation characterized by neurite outgrowth (dendrite and axon formation) on murine neuroblastoma N2a cells, which are cholinergic cells capable of differentiating into either cholinergic or dopaminergic cells depending on the culture conditions [35,36]. Interestingly, N2a cells express the PAC1 receptor, which is a member of the G-protein coupled receptor (GPCR) superfamily including the metabotropic receptors which bind octadecaneuropeptide (ODN) [37,38]. PAC1 and members of GPCR family activate adenylyl cyclase/cAMP/PKA (via Gs-protein coupling) and phospholipase C (PLC)/DAG/protein kinase C (PKC) (via Gq-protein coupling)-dependent signaling pathways [39,40]. The PAC1 receptor also triggers the activation of several other protein kinase cascades such as ERK1/2, JNK1/2, p38 MAPK and PKB [41–43]. Consequently, N2a cells have the ability to bind the pituitary adenylate cyclase-activating polypeptide (PACAP), which is widely distributed in the brain and peripheral organs and displays high affinity for the PAC1 receptor [40,44]. They can also be used to study other neuropeptides or molecules (natural or synthetic) capable of interacting with receptors of the GPCR superfamily. Thus, N2a cells constitute a suitable model to study neuronal differentiation and to identify the signaling pathways associated with this process. In this study, the effects of RSV and API on the neuronal differentiation of N2a were compared with those of trans-retinoic acid (RA) used as positive control. To this end, N2a cells were cultured without or with 10% FBS (conventional culture condition) since it is known that various factors present in FBS can modulate cell differentiation. Interestingly, RSV and API trigger the neuronal differentiation of N2a cells.

2. Materials and Methods

2.1. Cell Culture and Treatments

Mouse neuro-2a (N2a) neuroblastoma cells (ATCC® CCL-131™) were purchased from the American Type Culture Collection (Rockville, MD, USA). N2a cells were plated at a density of 34×10^3 cells/cm^2; they were cultured in Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/L), stable glutamine, and sodium pyruvate (Dominique Dutscher, Brumath, France) supplemented with 10% (v/v) fetal bovine serum (FBS, Pan Biotech, Aidenbach, Germany) and

containing 1% (*v*/*v*) antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin) (Pan Biotech). N2a cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere and passaged twice a week. N2a cells are broadly used to study the neuronal differentiation mechanism and neurite outgrowth [45].

To evaluate the cytotoxicity and the differentiating properties of resveratrol (RSV), apigenin (API), and retinoic acid (RA), 12×10^4 N2a cells were cultured per well in 6-well plates (FALCON, Becton Dickinson, NJ, USA) or in tissue culture dishes (35 × 10 mm, FALCON) containing 1 mL of culture medium with 10% FBS. After 24 h of cell culture, the culture medium was removed, and the cells were cultured for 48 h in the absence or presence of polyphenol (RSV, API) or RA used in a range of concentrations from 6.25 to 50 µM in culture medium without or with 10% FBS. RSV (corresponding to trans-resveratrol), API, and RA (corresponding to trans-retinoic acid) were from Sigma-Aldrich (St Quentin-Fallavier, France). Absolute ethanol (EtOH; Carlo Erba Reagents, Val de Reuil, France) was used as vehicle to dissolve RSV, whereas dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as vehicle to dissolve API and RA. RA was used as positive control to induce neuronal differentiation as it is well established that all-trans RA induce N2a differentiation into neuronal cells characterized by neurite outgrowth [46].

When the cells were simultaneously treated with the inhibitors H89 (20 µM), U73122 (1 µM), chelerythrine (1 µM) (Sigma-Aldrich), and U0126 (20 µM) (Calbiochem, San Diego, CA, USA), which contain PKA, PLC, PKC and MEK inhibitors, respectively, these compounds were introduced in the culture medium 30 min before RSV, API, or RA. H89 was prepared as a stock solution at 1 mM in distilled water; U0126, U73122 and chelerythrine were prepared as a stock solution in DMSO at 0.1 mM, 0.1 mM, and 1 mM, respectively. To evaluate the involvement of the metabotropic receptor in N2a cell differentiation, the metabotropic receptor antagonist $cyclo_{1-8}[DLeu^5]$ OP (10^{-6}M) was added in the culture medium 30 min before RSV, API, or RA.

2.2. Evaluation of Neuronal Differentiation with Morphological Criteria

Morphological criteria were used to evaluate neuronal differentiation [47–49] on N2a cells cultured in 6-well plates. These criteria were defined on N2a cells with the use of ODN, which is known to trigger neuronal cytoprotection and is capable of inducing differentiation on N2a cells. It is therefore considered as a neuroprotectin [50]. After 24 h of culture in DMEM with 10% FBS, the culture medium was removed and N2a cells were cultured for 48 h in the presence of very low concentrations of ODN (10^{-14} and 10^{-12} M) without FBS (0% FBS) or with 10% FBS. N2a cells (morphologically evocating neuroblasts) have the ability to differentiate in young immature and mature neurons. Under treatment with ODN, differentiated N2a cells with dendrites (D), axons (A) and dendrites + axons (D + A) were observed (Supplementary Figure S1).

These morphological criteria were further used to evaluate the ability of RSV and API to induce neuronal differentiation comparatively to RA used as a positive control. Morphological changes were evaluated after 48 h of culture, in DMEM without FBS (0% FBS), in the absence or presence of RSV (12.5 µM), API (12.5 µM), and RA (6.25 µM), and in DMEM with 10% FBS in the absence or presence of RSV (12.5 µM), API (12.5 µM) and RA (25 µM). Cells were observed by phase contrast microscopy under an inverted Zeiss microscope (Primovert, Jena, Germany) at a 20× magnification (Objective: LD Plan-Achromat, ref: 415500-1614-000). Digitalized images were obtained with a Zeiss camera (5MP HD IP). Neuronal differentiation was determined on 20 images corresponding to 20 microscopical fields (5 × 4) taken at the center of the culture dish. N2a cell differentiation was quantified by neurite outgrowth (dendrites and/or axons). The percentages of differentiated cells with dendrites, axons, and dendrites + axons were determined. All assays were performed at least in four independent experiments.

2.2.1. Cell Counting

N2a cells were cultured in 6-well plates. N2a cells previously cultured for 24 h in DMEM were further cultured for 48 h in DMEM without or with 10% FBS in the absence or presence of RSV, API,

and RA (6.25 to 50 μM). At the end of the treatment, adherent cells were collected by trypsinization and the total number of cells was determined by using a hemocytometer. All assays were realized at least in four independent experiments.

2.2.2. Measurement of Cell Viability with the Fluorescein Diacetate (FDA) Test

N2a cells, previously cultured for 24 h in DMEM with 10% FBS in 6-well plates, were further incubated for 48 h without or with 10% FBS in the absence or presence of RSV, API, and RA (6.25 to 50 μM). At the end of the treatment, cells were incubated for 10 min at 37 °C with 15 μg/mL fluorescein diacetate (FDA, Sigma-Aldrich), rinsed twice with phosphate buffer saline (PBS) and lysed with a Tris/HCl solution containing 1% sodium dodecyl sulfate (SDS) (Sigma-Aldrich). The FDA test is a cell viability test. Fluorescence was measured with excitation at 485 nm and emission at 528 nm using a plate reader (Tecan Sunrise, Tecan, Lyon, France). All assays were performed at least in four independent experiments.

2.2.3. Flow Cytometric Analysis of Cell Cycle

Flow cytometric analyses were carried out after staining with propidium iodide (PI) to determine the impact of RSV, API, and RA on the repartition of the cells in the different phases of the cell cycle. To this end, N2a cells previously cultured for 24 h in DMEM with 10% FBS were further cultured for 48 h either in DMEM without FBS, in the absence or presence of RSV (12.5 μM), API (12.5 μM), and RA (6.25 μM), or in DMEM with 10% FBS in the absence or presence of RSV (12.5 μM), API (12.5 μM), and RA (25 μM). At the end of the treatment, cell cycle was realized on adherent cells collected by trypsinization. Cells were washed with PBS, and 1–2 $\times$ 10^6 cells were stained with PI as previously described [51]. Cells were resuspended in 80% cold ethanol (2 h, −20 °C), washed in PBS, and resuspended in 300 μL of PBS containing 80 μg/mL PI and 200 μg/mL RNase A. After 1 h of incubation (37 °C, 1 h), 1–2 mL of PBS were added, and flow cytometric analyses were performed on a Galaxy flow cytometer (Partec, Münster, Germany). Fluorescence of PI was collected using a 590/10 nm bandpass filter and measured on a linear scale. Subsequently, 10,000 cells were acquired, and data were analyzed with Flomax software (Partec).

2.2.4. Real-Time PCR Analysis

Total RNAs from N2a cells obtained after 48 h of treatment with RSV (12.5 μM), API (12.5 μM) and RA (6.25 and 25 μM) without or with 10% FBS, were extracted and purified with the RNeasy Mini Kit (Qiagen, Courtaboeuf, France). The concentration of RNA was measured by spectrophotometry (UV-1800, Shimadzu, Kyoto, Japan) at an absorbance of 260 nm and calculated with UV Probe (Shimadzu software). Then, 1 μg of total RNA from each sample was converted into single-stranded cDNA using the iScript cDNA kit (BioRad, Marne la Coquette, France) according to the protocol reaction provided by the manufacturer: 5 min at 25 °C, 20 min at 46 °C, and 5 min at 95 °C. cDNA was then amplified in the presence of FG Power SYBR Green (Thermo Fischer Scientific, Illkirch-Graffenstaden, France) and 100 μM of forward and reverse mouse primers (Eurogentec, Liége, Belgium):

* Diazepam-binding inhibitor (DBI) sequences: forward (*5′-gaagcgcctcaagactcagc-3′*) and reverse (*5′-ttcagcttgttccacgagtcc-3′*),

* Nerve growth factor (NGF) sequences: forward (*5′-acactctgatcactgcgtttttg-3′*) and reverse (*5′-ccttctgggacattgctatctgt-3′*),

* Brain-derived neurotrophic factor (BDNF) sequences: forward (*5′-atggttatttcatacttcggttgca-3′*) and reverse (*5′-agctgggtaggccaagttg-3′*),

* 36B4 sequences (*36B4* was used as reference gene): forward (*5′-gcgacctggaagtccaacta-3′*) and reverse (*5′-atctgcttggagcccacat-3′*)

StepOne Plus (Life Technologies/Thermo Fischer Scientific, Courtaboeuf, France) was used to detect the real-time quantitative PCR products of reverse-transcribed cDNA samples according to the

manufacturer's instructions. The incubation conditions were as follows: 90 °C for 20 s, followed by 40 cycles (95 °C for 30 s, 60 °C annealing for 30 s) and a cycle from 60 °C to 95 °C. Specific amplification efficiencies were calculated with StepOne software. Results were expressed as means of Ct (threshold cycle value) ± standard deviation (SD).

2.2.5. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism5 software (San Diego, CA, USA). An ANOVA test followed by a Mann–Whitney test was used. A p value of 0.05 or less were considered as statistically significant.

3. Results

3.1. Evaluation and Quantification of Resveratrol- and Apigenin-Induced Neuronal Differentiation of N2a Cells

N2a cells were previously cultured for 24 h in the following culture medium: DMEM high glucose (4.5 g/L) with stable glutamine and sodium pyruvate containing 10% FBS. They were further cultured for 48 h in DMEM with high glucose and stable glutamine and sodium pyruvate without FBS (0% FBS), or with 10% FBS in the absence or in the presence of RSV (6.25–50 μM) or API (6.25–50 μM). Ethanol (EtOH) was used as vehicle to dissolve RSV whereas DMSO was used as vehicle to dissolve API and RA (6.25–50 μM) used as positive control to induce neuronal differentiation. Neuronal differentiation induced by RSV, API, and RA was morphologically evaluated by phase contrast microscopy on 20 images taken with a Zeiss camera under an inverted Zeiss microscope (Figure 1). In those conditions, cell differentiation was evaluated by neurite outgrowth (dendrites and/or axons) and differentiated cells with dendrites, axons, and dendrites + axons were quantified. In the different conditions of culture, the spontaneous level of total differentiated cells (cells with dendrites, axons, and dendrites + axons) was similar with 0% FBS and with 10% FBS (Figure 2).

Without FBS (0% FBS), the highest percentage of differentiated cells was observed with RA (6.25 μM; 53 ± 9%) (Figure 2); at the highest concentrations, lower percentages of differentiated cells were detected (Figure 2): this could be due to a dose-dependent decrease of cell growth and/or viability induced by RA revealed by cell counting and staining with fluorescein diacetate (FDA), respectively (Figure 3A,B). In the presence of RSV and API, the neuronal differentiation was in the same range of order (28–40%) from 6.25 to 50 μM (Figure 2); at these concentrations, cell growth and cell viability was sometimes slightly but significantly increased with API (Figure 3A,B) whereas a significant decrease in cell growth and viability was found with RSV (6.25 and 12.5 μM) (Figure 3A,B).

Interestingly, with 10% FBS, the highest percentages of differentiated cells (30–38%) associated with a decrease of cell growth and viability were observed with RSV in a range of concentration from 12.5 to 50 μM (Figures 2 and 3A,B). Similar differentiating effects (10–30%), in a range of concentration from 12.5 to 50 μM, without or with a slight cytotoxicity, were observed with API and RA (Figures 2 and 3A,B).

Under treatment with RSV, and especially with API and RA, as the induction of differentiation, as well as the impact on cell growth and viability were different without FBS and with 10% FBS (the differentiation was often reduced in the presence of 10% FBS), N2a cells were treated with RSV, API, and RA in the presence of 10% delipidized FBS to determine the incidence of the lipidic and non-lipidic fraction of the serum on differentiation. Of note, in the presence of 10% delipidized FBS, the differentiating effects of RSV, API, and RA were strongly reduced, supporting that the inhibiting effects of the FBS on neuronal differentiation are due to non-lipidic compounds (Figure 2).

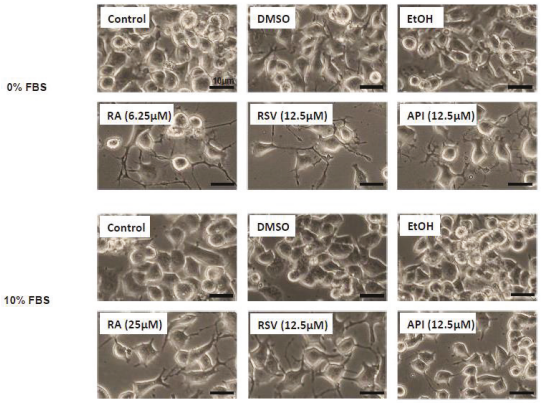


Figure 1. Morphological evaluation of neuronal differentiation of neuro-2a (N2a) cells treated with resveratrol, apigenin, and retinoic acid used as positive control. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without or with 10% fetal bovine serum (FBS) in the absence or in the presence of retinoic acid (RA: 6.25 and 25 μM) respectively, used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV 12.5 μM) and apigenin (API 12.5 μM). Control cells were cultured in medium without or with 10% FBS. Two vehicle controls were realized: ethanol (EtOH) used with RSV, and dimethyl sulfoxide (DMSO) used with RA and API. Observations were realized by phase contrast microscopy. Differentiated cells, characterized by neurite outgrowth (dendrites and/or axons), are observed in the presence of RSV, API, and RA.

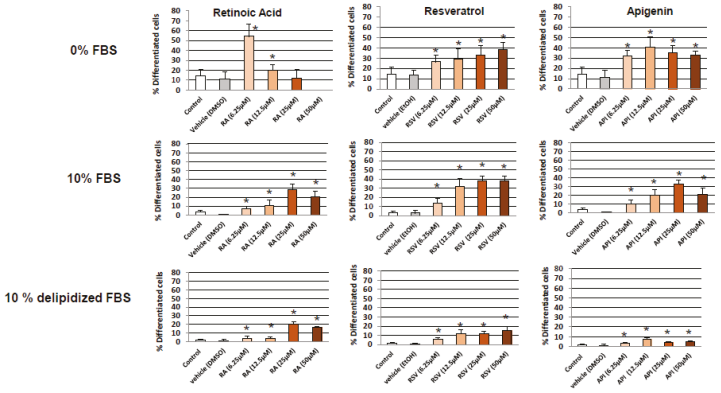


Figure 2. Quantification of neuronal differentiation induced by resveratrol and apigenin on N2a cells. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without or with 10% FBS in the absence or in the presence of retinoic acid (RA: 6.25–50 μM) used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 6.25–50 μM) and apigenin (API: 6.25–50 μM). Control cells were cultured in medium without and with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, and DMSO used with RA and API. The percentages of differentiated cells include cells with dendrites, axons and dendrites + axons; these percentages were determined by phase contrast microscopy. Each value is the mean ± standard deviation (SD) of four independent experiments. An ANOVA test followed by a Mann-Whitney test were used. p values of 0.05 or less were considered as statistically significant. * comparison between control (untreated cells), vehicles (DMSO, Ethanol (EtOH)), and RSV, API and RA; no difference between control/vehicles (DMSO, EtOH) was observed.

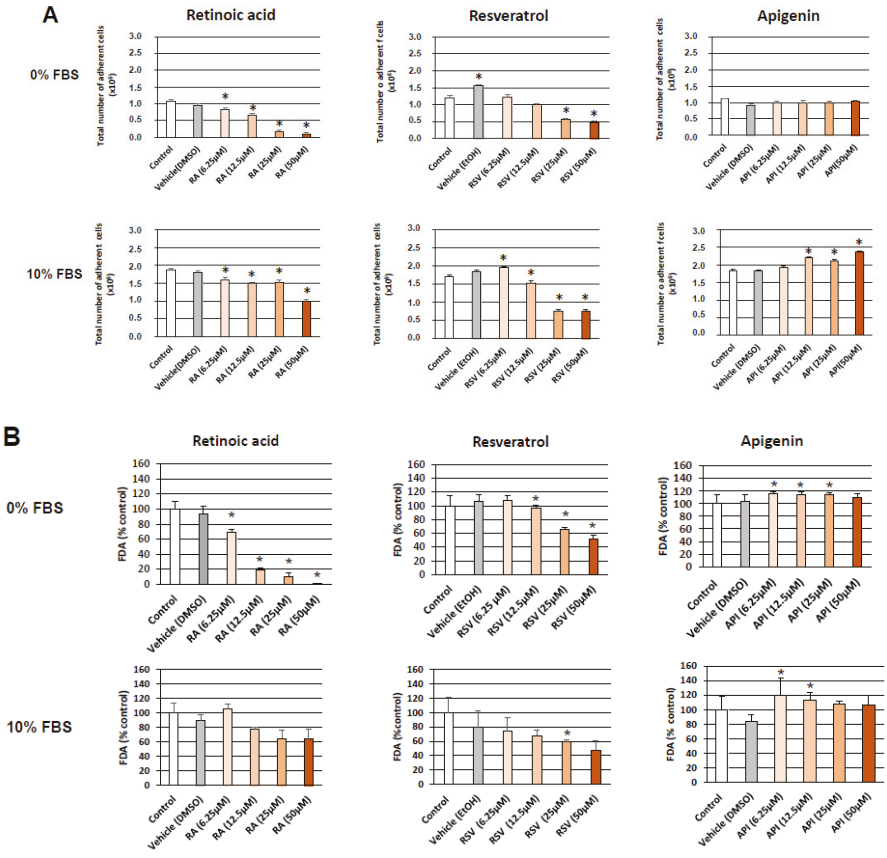


Figure 3. Incidence of neuronal differentiation induced by resveratrol and apigenin on cell growth and viability of N2a cells. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without and with 10% FBS in the absence or in the presence of retinoic acid (RA: 6.25–50 µM) used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 6.25–50 µM) and apigenin (API: 6.25–50 µM). Control cells were cultured in medium without and with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, and DMSO used with RA and API. Cell growth (total number of adherent cells) was determined by cell counting (**A**) and cell viability by fluorimetry with the FDA test (**B**). Each value is the mean ± standard deviation (SD) of four independent experiments. An ANOVA test followed by a Mann–Whitney test were used. *p* values of 0.05 or less were considered as statistically significant. * comparison between control (untreated cells), vehicles (DMSO, EtOH), and RSV, API and RA; no difference between control/vehicles (DMSO, EtOH) was observed.

Preliminary data on the repartition of the cells in the different phases of the cell cycle have been obtained (Supplementary Figure S2A,B). Without FBS (0% FBS), slight modifications of the cell cycle were observed compared to the control and vehicle-treated cells in the presence of RSV, API, and RA used at a concentration inducing differentiation. With 10% FBS at concentrations inducing differentiation, modifications of the cell cycle were mainly observed with RSV (12.5 µM). However, at a concentration of 25–50 µM, which is used to induce cell death (especially the 50-µM concentration), RSV and API induce modifications of the cell cycle as previously reported. Thus, RSV (25–50 µM), in agreement with other studies [51,52], induces an accumulation of the cells in the S

phase. API (25–50 μM) triggers a less pronounced accumulation of the cells in the S phase than that observed with RSV, whereas its ability to favor an accumulation in G2 + M has been reported [53,54]. RA induces an accumulation of the cells in G2 + M.

For further experiments without FBS and with 10% FBS, the concentrations of RSV, API, and RA that were chosen are a compromise in terms of the ability to induce high differentiation without or with a low toxicity. The lowest concentrations meeting these criteria were selected: RSV and API, 12.5 μM without and with 10% FBS; and RA, 6.25 and 25 μM, without and with 10% FBS, respectively. In those conditions, RSV and API induce neurite outgrowth, and N2a cells with dendrites, axons, and dendrites + axons were observed (Figure 4). Depending on the compound considered the percentage of cells with dendrites or axons was more or less different without or with 10% FBS (Figure 4). The highest percentage of axonal cells (20–50%) which can be considered as a sign of terminal neuronal differentiation was observed with RA, whereas the percentage of axonal cells was from 22 to 30% and 16 to 34% with RSV and API, respectively (Figure 4; Supplementary Figure S3).

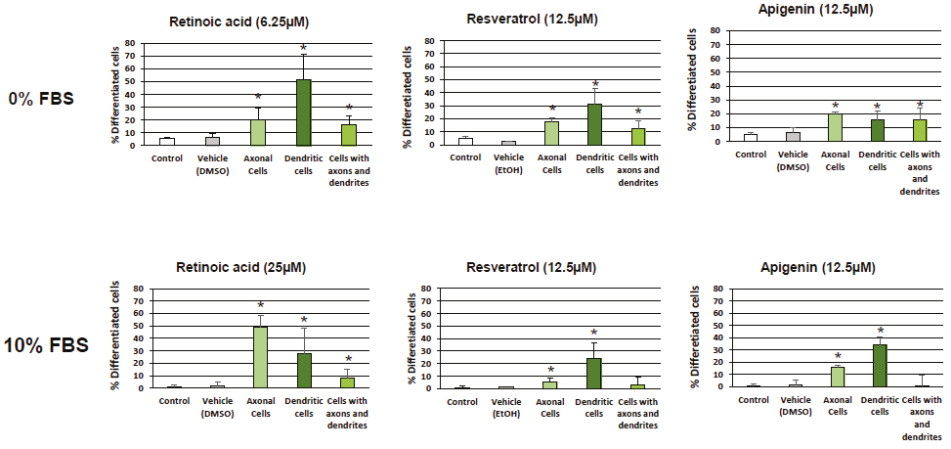


Figure 4. Characterization and quantification of differentiated neuronal cells (cells with dendrites, axons and dendrites + axons) induced by resveratrol and apigenin. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without and with 10% FBS in the absence or in the presence of retinoic acid (RA: 6.25 μM with 0% FBS; 25 μM with 10% FBS) used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 12.5 μM with 0% and 10% FBS) and apigenin (API: 12.5 μM with 0% and 10% FBS). Control cells were cultured in medium without and with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, and DMSO used with RA and API. The percentages of cells with dendrites, axons, and axons + dendrites are shown, and were determined by phase contrast microscopy. Each value is the mean ± standard deviation (SD) of four independent experiments. An ANOVA test followed by a Mann–Whitney test were used. p values of 0.05 or less were considered as statistically significant. * comparison between control (untreated cells), vehicles (DMSO, EtOH), and RSV, API, and RA; no difference between control/vehicles (DMSO, EtOH) was observed.

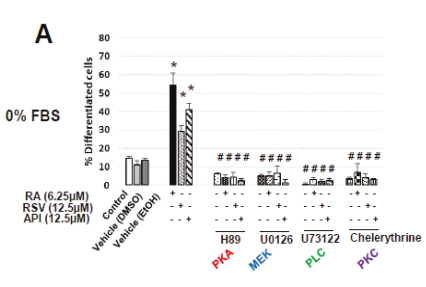
Altogether, these data show that RSV and API are potent inducers of neuronal differentiation, inducing neuritis and axon outgrowth (terminal neuronal differentiation). Of note, at the opposite of RA and RSV, these differentiating effects of API were associated with no or slight cytotoxic effects as shown by cell counting and the FDA test.

3.2. Characterization of Resveratrol- and Apigenin-Signaling Pathways Involved in the Neuronal Differentiation of N2a Cells

At the moment, little is known about the signaling pathways involved in the neuronal differentiation induced by neurotrophins. As octadecaneuropeptide (ODN), which can be considered as a neurotrophin, has cytoprotective effects via metabotropic receptors involving both the activation of the PKA/(PLC/PKC)/(MEK/ERK) signaling pathway [50], we determined whether PKA, PLC, PKC and MEK/ERK were involved in the neuronal differentiation induced by RSV and API (as well as of RA used as positive control) with different inhibitors: H89 (PKA), U0126 (MEK), U73122 (PLC) and chelerythrine (PKC).

Without FBS (0% FBS), when RSV and API were associated with H89, U0126, U73122, and chelerythrine, a total inhibition of neuronal differentiation (% of total differentiated cells: cells with dendrites and/or axons) was observed (Figure 5A). This supports that these two polyphenols simultaneously stimulate the following signaling pathways PLC/PKC/(MEK/ERK) and PKA/MEK, or that they act via a receptor evocating the canonical metabotropic receptors which simultaneously involves the activation of PKA and PLC/PKC, leading to activation of MEK/ERK (Figure 5A).

With 10% FBS, important modifications in the signaling pathways activated by RSV and API were observed. With RSV, PLC/PKC as well as PKA were required for the induction of neuronal differentiation (Figure 5B). With API, the neuronal differentiation was independent of PKA, PLC/PKC, and MEK/ERK (Figure 5B). With retinoic acid, PLC/PKC were only required for the induction of neuronal differentiation (Figure 5B). Thus, with RSV, PLC/PKC were activated both with 0% and 10% FBS; with API, PKA, PLC/PKC, and MEK/ERK were activated with 0% FBS, whereas none of these pathways were needed with 10% FBS; RA, PKA, and PLC/PKC were activated both with 0% and 10% FBS (Figure 5A,B).



	PKA (H89)	MEK (U0126)	PLC (U73122)	PKC (Chelerythrine)
RA	ID	ID	ID	ID
RSV	ID	ID	ID	ID
API	ID	ID	ID	ID

*ID: Inhibition of differentiation
*NID: No inhibition of differentiation

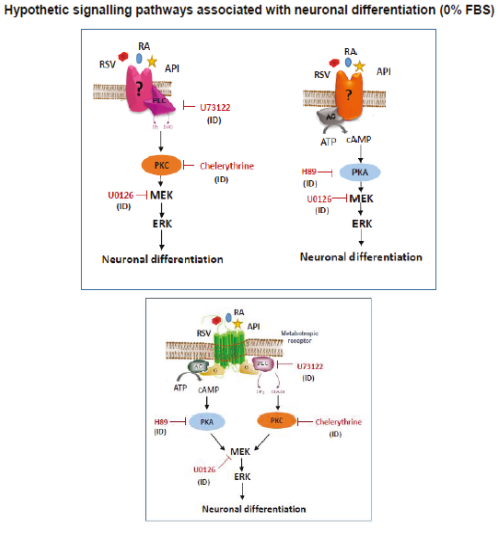
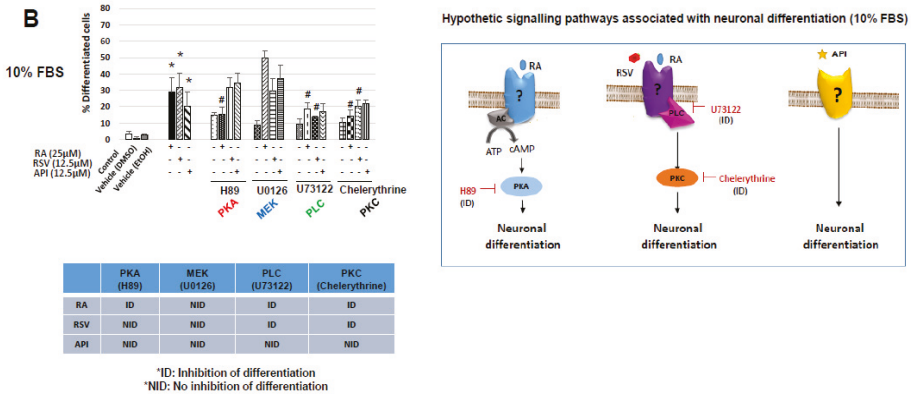


Figure 5. *Cont.*



	PKA (H89)	MEK (U0126)	PLC (U73122)	PKC (Chelerythrine)
RA	ID	NID	ID	ID
RSV	NID	NID	ID	ID
API	NID	NID	NID	NID

*ID: Inhibition of differentiation
*NID: No inhibition of differentiation

Figure 5. Evaluation of protein kinase A (PKA), phospholipase C (PLC)/protein kinase C (PKC) and the MEK/ERK signaling pathways in resveratrol- and apigenin-induced neuronal differentiation of N2a cells cultured without FBS (0% FBS) and with 10% FBS. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without FBS (0% FBS) (**A**) or with 10% FBS (**B**) in the absence or in the presence of retinoic acid (RA: 6.25, 25 µM) respectively, used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 12.5 µM) and apigenin (API: 12.5 µM). RSV and API were used either without or with different inhibitors: H89 (2×10^{-5} M; PKA), U0126 (10^{-6} M; MEK), U73122 (10^{-7} M; PLC) and chelerythrine (10^{-7} M; PKC) [50]. Control cells were cultured in medium without FBS (0% FBS) or with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, and DMSO used with RA and API. Control cells and vehicle-treated cells were also cultured without or with H89, U0126, U73122 and chelerythrine. The percentages of differentiated cells were determined by phase contrast microscopy. The hypothetic signaling pathways associated with RSV, API, and RA without FBS (0% FBS) and with 10% FBS are represented on the right of the corresponding Figures and Tables. The values are means ± standard deviation (SD) of four independent experiments. An ANOVA test followed by a Mann–Whitney test were used. p values of 0.05 or less were considered as statistically significant. * comparison between control (untreated cells), vehicles (DMSO, EtOH), and RSV, API, and RA; # comparison between RSV, API, and RA, and RSV, API, and RA associated with H89, U0126, U73122, or chelerythrine.

3.3. Evaluation of the Involvement of Octadecaneuropeptide (ODN) Receptor in the Neuronal Differentiation of N2a Cells Induced by Resveratrol and Apigenin

Based on the data obtained with RSV and API without FBS (0% FBS), an involvement of the metabotropic receptors of ODN can be suspected. We therefore simultaneously cultured N2a cells with RSV and API, without and with cyclo$_{1-8}$[DLeu^5] OP (an antagonist ODN metabotropic receptor) [50]. In those conditions, the neuronal differentiation induced by RSV and API was not reduced (Figure 6) supporting that the direct activity of RSV and API does not require the metabotropic receptors of ODN, or that RSV and API do not activate the synthesis of DBI, the precursor of ODN [37]. In agreement with this later hypothesis, in comparison to untreated and vehicle-treated cells, no increase of DBI mRNA levels evaluated with the Ct values were observed by RT-qPCR when N2a cells were cultured with resveratrol and apigenin; similar data were found with retinoic acid (Table 1).

In addition, under treatment with RSV and API, no increase of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) mRNA levels evaluated with the Ct values was observed by RT-qPCR (Table 1). Similarly, no effect of RA, on DBI, NGF, and BDNF mRNA level was found (Table 1).

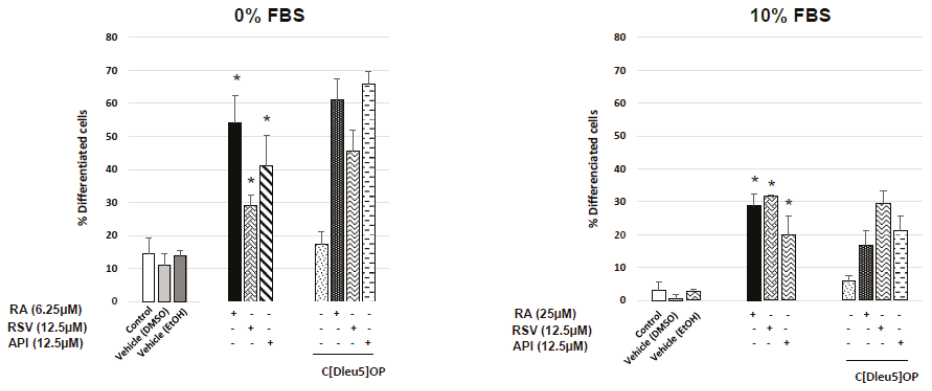


Figure 6. Evaluation of the implication of the ODN receptor in neuronal differentiation induced by resveratrol and apigenin on N2a cells. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without or with 10% FBS in the absence or in the presence of retinoic acid (RA: 6.25 µM with 0% FBS; 25 µM with 10% FBS) used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 12.5 µM with 0% and 10% FBS) and apigenin (API: 12.5 µM with 0% and 10% FBS). RSV and API were cultured either without or with $Cyclo_{1-5}$[$Dleu^5$] OP (10^{-6} M; an antagonist of ODN metabotropic receptor) [50]. Control cells were cultured in medium without and with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, and DMSO used with RA and API. Control cells and vehicle-treated cells were also cultured without or with $Cyclo_{1-5}$[$Dleu^5$] OP. The percentages of differentiated cells were determined by phase contrast microscopy. The values are means ± standard deviation (SD) of four independent experiments. An ANOVA test followed by a Mann–Whitney test were used. p values of 0.05 or less were considered as statistically significant. * comparison between control (untreated cells), vehicles (DMSO, EtOH), and RSV, API, and RA; no significant differences were observed between RSV, API and RA, and RSV, API, and RA associated with C[$Dleu^5$] OP. No difference was observed between control/vehicles (DMSO, EtOH) or between control/C[$Dleu^5$] OP.

Table 1. Effects of resveratrol, apigenin, and retinoic acid on the Ct values of neuropeptides with potential neurotrophin activities: diazepam binding inhibitor (DBI; the precursor of octadecaneuropeptide (ODN)), nerve growth factor (NGF) and brain derived neurotrophic factors (BDNF).

Neuropeptides	% FBS	Ct					
		Control	EtOH (0.02 %)	DMSO (0.12 %)	RA	RSV	API
DBI	**0 %**	33.5 ± 0.1	33.5 ± 1.6	30.6 ± 0.3	33.6 ± 1.0	33.4 ± 1.2	31.9 ± 0.1
	10 %	30.8 ± 0.5	28.4 ± 0.3	28.2 ± 0.3	32.2 ± 0.2	28.9 ± 2.4	28.1 ± 0.5
NGF	**0 %**	32.9 ± 3.7	31.3 ± 1.3	28.1 ± 0.6	29.7 ± 0.2	30.0 ± 0.3	28.5 ± 0.1
	10 %	27.8 ± 0.5	25.4 ± 1.6	25.6 ± 1.8	28.6 ± 0.2	24.9 ± 0.2	25.3 ± 0.2
BDNF	**0 %**	34.8 ± 0.8	33.4 ± 1.1	29.7 ± 1.1	32.6 ± 0.7	35.5 ± 2.3	31.3 ± 1.7
	10 %	31.0 ± 0.6	28.1 ± 0.2	27.4 ± 0.5	31.1 ± 0.5	37.6 ± 0.5*	27.6 ± 0.1

Ct values were obtained by RT-qPCR on N2a cells. N2a cells were previously cultured for 24 h in conventional cultured medium; they were further cultured for 48 h in medium without or with 10% FBS in the absence or in the presence of retinoic acid (RA: 6.25 µM with 0% FBS; 25 µM with 10% FBS) used as positive control for the induction of neuronal differentiation, or with polyphenols: resveratrol (RSV: 12.5 µM with 0% and 10% FBS) and apigenin (API: 12.5 µM with 0% and 10% FBS). Two vehicle controls were realized: ethanol (EtOH) used with RSV, DMSO used with RA and API. Data shown are the mean ± SD of two independent experiments realized in triplicate. With the Mann-Whitney test, for DBI, NGF and BDNF, no significant differences were observed between control, ETOH (0.02%) and DMSO (0.12%). For DBI and NGF, no significant differences were found between RA, RSV, API and the corresponding vehicles. For BDNF, no significant differences were found between RA, RSV, API and the corresponding vehicles, excepted with RSV (10% FBS; * $p \leq 0.05$%).

4. Discussion

Polyphenols are a broad family of molecules including flavonoids, phenolic acids, lignans and stilbenes (such as resveratrol) [3,4]. Flavonoids include flavones (such as apigenin), flavonols, flavanones, flavanols, isoflavones, and anthocyanins. The polyphenols are found in large quantities in the Mediterranean diet which is rich in fruits and vegetables and which can be associated with a consumption of wine [55]. Polyphenols are for the most part powerful antioxidants, and some of them, such as apigenin and resveratrol, also have anti-proliferative and anti-inflammatory properties and decrease the production of growth factors as insulin growth factor-1 (IGF-1) and vascular endothelial growth factor (VEGF) [15,56]. With resveratrol, pro-differentiating activities have also been shown on myoblasts, resulting in the formation of myotubes [57]. Apigenin has also been shown to promote osteogenic differentiation of human mesenchymal stem cells through JNK and p38 MAPK pathways [58,59], to enhance myoblast differentiation by regulating Prmt7 [60], to induce granulocytic differentiation in human promyelocytic leukemia HL-60 cells [61], and to activate morphological differentiation and G2-M arrest in rat neuronal cells [62]. It has also been reported that apigenin from Croton betulaster Mull inhibits proliferation, induces differentiation and regulates the inflammatory profile of C6 glioma cells [63].

In response to the increase in age-related diseases, particularly neurodegenerative diseases associated with oxidative stress and inflammation [5], polyphenols, because of their anti-oxidant and anti-inflammatory activities, could be used for prevention or even as a treatment. Several studies also reveal a benefit of polyphenols to cognitive functions [22]. Moreover, it is now well established that resveratrol prevents the aggregation of β-amyloid, which is neurotoxic [64]. In the more common neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, neuronal loss is associated with the evolution of these diseases. Promoting neurogenesis could be a means of preventing these diseases. In this context, neuropeptides produced by the brain via endothelial cells and astrocytes form a family of molecules called neurotrophins, with cytoprotective and differentiating activities [65]. However, to exert their cytoprotective effects, these molecules must be injected intracerebrally near the lesions [66]. As polyphenols have the ability to cross the blood–brain barrier and accumulate in the brain much more efficiently when administered intravenously than orally [27,67,68], these properties reinforce their interest in preventing certain neurodegenerative diseases.

In this context, it was therefore important to clarify whether some polyphenols (resveratrol, apigenin) present in significant amount in the Mediterranean diet could promote neurogenesis, especially the differentiation of neuroblasts into mature neurons characterized by the presence of dendrites and/or axons. Using N2a murine neuroblastoma cells, we demonstrated that these cells cultured in the presence of resveratrol and apigenin in a concentration range from 6.25 to 50 μM differentiate into mature neurons with dendrites and/or axons and that the activation of the PKC signaling pathway plays an important role in this process. These results, which establish that some polyphenols (resveratrol, apigenin) are able to trigger neuronal differentiation, opening new perspectives in terms of treatments for neurodegenerative diseases where only few molecules are effective.

Interestingly, the differentiation observed with resveratrol and apigenin on N2a cells is as effective as that obtained with retinoic acid (used as positive control), which in humans can be associated with various side effects because of its ability to activate or suppress the expression of many genes [69]. In the present study, in terms of toxicity, the differentiation induced by resveratrol as well as with retinoic acid is associated with an inhibition of cell growth resulting from a decrease of cell viability, as evaluated with the FDA test. On the other hand, with apigenin, neuronal differentiation has only little effect on cell growth and viability, suggesting different mechanisms of differentiation between resveratrol and apigenin. In addition, the differentiation observed in the absence of FBS (0% FBS) demonstrates that exogenous factors present in the serum do not contribute to the neuronal differentiation induced by resveratrol and apigenin as well as with retinoic acid. Moreover, a comparison of the efficiency of resveratrol and apigenin to induce neuronal differentiation in serum-free culture medium (0%

FBS) and in culture medium containing 10% FBS clearly shows that FBS attenuates the differentiation induced by the polyphenols and retinoic acid, and that FBS also contributes to the attenuation of the cytotoxicity, mainly with retinoic acid. As the comparison of the differentiation obtained in the presence of delipidized serum (10% delipidized FBS) versus 10% FBS shows a lower differentiation in the presence of 10% delipidized FBS than in the presence of 10% FBS, our results demonstrate that some serum proteins counteract neuronal differentiation. The identification of these molecules could make it possible to optimize the use of polyphenols for neurodifferentiation purposes in humans. The fractionation of the serum associated with a proteomic analysis must make it possible to answer this question. It has been reported that serum factors such as α_1-, α_4-, and β-globulin fractions can cause the inhibition of neuronal differentiation and neurite growth [70–72], whereas serum deprivation increases the phosphorylation of EGFR, ERK1/2, AKt, and other signaling molecules in N2a cells [72]. On the basis of in vitro tests using patient serum instead of FBS, one can also expect to distinguish between good and bad responders to treatment with polyphenols.

In addition, as the ability of RSV, API, and RA to trigger neuronal differentiation occurs without and with 10% FBS, and is associated with modifications of the cell cycle (RSV and API: marked and slight accumulation of the cells in the S phase of the cell cycle, respectively; RA: accumulation of the cells in the G2 + M phase of the cell cycle), the role taken by cell cycle-associated molecules in the neuronal differentiation of N2a cells will require additional investigation.

Altogether, these data underline that in order to promote neurogenesis with polyphenols, it is necessary to identify the signaling pathways involved in this process to develop effective drugs and to optimize the efficiency of resveratrol and apigenin. Hence, we tried to specify which signaling pathways were activated with resveratrol and apigenin but also with retinoic acid used as a positive control.

Due to the ability of certain neurotrophic factors (characterized by cytoprotective and differentiating properties) such as ODN to exert their activities via metabotropic receptors (G protein-coupled receptors) [73], some molecules capable of inhibiting the metabotropic receptor-associated response under the effect of ODN were used: H89 (PKA inhibitor), U0126 (MEK inhibitor), U73122 (PLC inhibitor), and chelerythrine (PKC inhibitor) [38,50]. Without FBS, when RSV and API were associated with H89, U0126, U73122, and chelerythrine, a total inhibition of neuronal differentiation was observed supporting either that these two polyphenols are able to simultaneously stimulate the PLC/PKC/(MEK/ERK) and PKA/(MEK/ERK) signaling pathways, or that they act via a receptor evocating the metabotropic receptors which simultaneously involves the activation of PKA and PLC/PKC, leading to the activation of MEK/ERK. However, with 10% FBS, important modifications in the signaling pathways activated by RSV and API were observed. With RSV, PLC/PKC as well as PKA were activated, whereas with API, the neuronal differentiation was independent of PKA, PLC/PKC, and MEK/ERK. Thus, with RSV, the PLC/PKC signaling pathway was activated both with 0% and 10% FBS, whereas with API, PKA, PLC/PKC, and MEK/ERK were activated with 0% FBS. None of these pathways were required with 10% FBS. These data bring new evidence supporting that the biological activities of RSV and API (impact on cell growth, viability, and differentiation) involve different mechanisms. With RA (used as positive control), PKA and PLC/PKC were activated both with 0% and 10% FBS. Altogether, these data support that the early metabolic pathways involved in neuronal differentiation depend on the inducer considered, and that the differentiating activities of polyphenols (RSV, API) could involve plasma membrane receptors.

In the absence of serum, as the implication of a receptor evoking the metabotropic receptor and simultaneously activating the PKC and PKA pathways was considered [73,74], we attempted to determine whether RSV and API were able to activate the synthesis of DBI, which is an ODN precursor [37]. In those conditions, the ODN produced could in turn activate the metabotropic receptor in an autocrine or paracrine way. The very low values of Ct obtained for DBI in the control cells as well as in the cells cultured in the absence or presence of RSV and API, without FBS or with 10% FBS,

excludes this hypothesis. In addition, no effects of RSV and API were observed on NGF and BDNF mRNA levels.

As RSV and API display more ability to differentiate N2a cells than RA, we suggest the following potential mechanism behind this phenomenon. The growth of dendrites and/or neurons requires much energy and mitochondrial biogenesis [75]. In addition, dietary micronutrients (including polyphenols) have been shown to favor mitochondrial/nuclear dialogue, which could favor gene expression such as in those involved in neuronal differentiation [76]. In well-differentiated neuronal cells, a topographical redistribution of mitochondria along the axone is also needed to favor the transmission of the nerve impulse [77]. As several polyphenols are recognized as molecules capable of modulating pathways involved in mitochondrial biogenesis (induction of sirtuins), mitochondrial activity (modulating complexes I to V activity, ATP production), and control of the intra-mitochondrial oxidative status (inhibition of ROS formation), the different benefits of this family of compounds (including RSV and API) at the mitochondrial level might be predominant [78]. In addition, the mitochondria also play key roles in lipid metabolism, especially fatty acids, which are required for the biogenesis of lipids (fatty acids, phospholipids) present in the membrane of dendrites and neurons [79]. In contrast, RA is rather known to favor mitochondrial permeability and transition, leading to apoptosis [80]. It is therefore suggested that the different impacts of RSV, API, and RA at the mitochondrial level, and also probably in other organelles (lysosome, peroxisome) playing key roles in the control of lipid metabolism and the equilibrium between life and death might contribute, at least in part, to the different neuronal differentiation capacities of these different molecules.

5. Conclusions

Our data obtained on murine neuroblastoma N2a cells establish that RSV and API are able to induce neuronal differentiation and favor neurogenesis characterized by neurite outgrowth. Thus, when treated with RSV and API, the differentiated N2a cells are characterized by the presence of dendrites and axons and less frequently by the simultaneous presence of dendrites + axons. These morphological criteria demonstrate that RSV and API trigger a neuronal differentiation inducing the formation of mature neurons. Since polyphenols also exhibit cytoprotective, mainly anti-oxidant properties on neurodegeneration models [81,82], their cytoprotective and neuron-differentiating properties suggest that these compounds may mimic neurotrophin activities. These data support that micronutrients such as RSV and API, which are widely represented in the Mediterranean diet, could be of interest for the prevention and/or the treatment of neurodegenerative diseases associated with neuronal loss (Alzheimer's disease, Parkinson's disease). These data also reinforce the interest of polyphenols for the treatment of major aged-related diseases associated with neurodegeneration (Alzheimer's disease, Parkinson's disease) for which the therapeutic arsenal is reduced.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-9721/6/3/67/s1, Supplementary Figure S1: Morphological criteria allowing discrimination between cells with dendrites, axons, and dendrites + axons. Murine neuronal N2a cells previously cultured for 24 h were further cultured for 48 h in medium without FBS (0% FBS) or with 10% FBS in the presence of ODN (10^{-14} and 10^{-12} M) used as neuronal differentiation inducer. In the presence of ODN, N2a cells were differentiated in neurons with dendrites (D), axons (A), and dendrites + axons (A + D). These morphological criteria were used to quantify the neuronal differentiation induced by RSV, API, and RA. Supplementary Figure S2: Impact of resveratrol, apigenin and retinoic acid on the repartition of the cells in the different phases of the cell cycle when cultured without or with 10% fetal bovine serum. Murine neuronal N2a cells previously cultured for 24 h in conventional cultured medium were further cultured for 48 h in medium without or with 10% FBS in the absence or in the presence of RA (6.25, 12.5 and 25 μM) used as positive control for the induction of neuronal differentiation, or with polyphenols: RSV (12.5, 25 and 50 μM), and API (12.5, 25 and 50 μM). Cell cycle analysis was performed by flow cytometry after staining with propidium iodide. Preliminary data shown correspond to one experiment. The ability of RSV (25 and 50 μM) to increase the percentage of cells in the S phase of the cell cycle validates the experiment. A—Histograms obtained by flow cytometry illustrating the repartition of N2a in the different phases of the cell cycle under treatment with RSV, API, and RA in culture medium without or with 10% FBS; B—percentages of cells in the different phases of the cell cycle (N2a cells treated with RSV, API and RA in culture medium without or with 10% FBS. Supplementary Figure S3: Morphological aspect of N2a cells cultured without or with 10% FBS in the presence of retinoic acid, resveratrol, or apigenin. Murine neuronal N2a cells previously cultured for 24 h in conventional

cultured medium were further cultured for 48 h in medium without and with 10% FBS in the absence or in the presence of RA (6.25 µM with 0% FBS; 25 µM with 10% FBS) used as positive control for the induction of neuronal differentiation, or with polyphenols: RSV (12.5 µM with 0% and 10% FBS) and API (12.5 µM with 0% and 10% FBS). Control cells were cultured in medium without and with 10% FBS. Two vehicle controls were realized: EtOH used with RSV, DMSO used with RA and API. The supplementary Figure S3 illustrates the data presented in Figure 4. The morphological aspect of N2a cells cultured without or with 10% FBS in the presence of retinoic acid, resveratrol, or apigenin is shown. The pictures were obtained by phase contrast microscopy.

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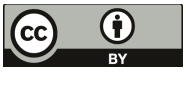

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Grape Pomace: Antioxidant Activity, Potential Effect Against Hypertension and Metabolites Characterization after Intake

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Abstract: Observational studies indicate that the intake of polyphenol-rich foods improves vascular health, thereby significantly reducing the risk of hypertension and cardiovascular disease (CVD). Therefore, the aim of this study was to analyse the remained potential of grape by-products from important Rhône Valley red wine cultivars: Grenache, Syrah, Carignan, Mourvèdre and Alicante. For that, six different extracts from grape pomaces, selected by their antioxidant activity, were studied in vivo during six weeks with spontaneously hypertensive rats (SHR). Extracts used in SHR1, SHR2 and SHR6 groups presented a « rebound effect » on systolic blood pressure, whereas the other extracts do not change it significantly. The bioavailability of Grenache (GRE1) (EA70) seed pomace extract (SHR1 group), Mouvendre (MOU) (EA70) skin pomace extract (SHR5 group) and Alicante (ALI) (EA70) skin pomace extract (SHR6 group) was studied by High Performance Liquid Chromatography with Photodiode Array detector and Electrospray Ionization Mass Spectrometer (HPLC-PDA-ESI-MS^n) in urine, plasma and tissues to search differences on the metabolism of the different extracts intake.

Keywords: grape pomace; polyphenols; hypertension; metabolites characterization

1. Introduction

Polyphenols are the most abundant and ubiquitous secondary metabolites present in the plant kingdom with more than 8000 phenolic structures currently known. These compounds play an important role in plant growth and reproduction, providing protection against biotic and abiotic stress such as pathogen and insect attack, ultra violet (UV) radiation and wounding [1,2].

Dietary intake of (poly)phenols has been estimated to be about 1 g/day [3]. Their intake is 10 times greater than that of vitamin C and 100 times that of vitamin E or the carotenoids [4]. As a result, phenolic compounds are currently receiving much attention because of their favourable health effect related to their antioxidant properties. Indeed, several studies have focused their attention on the components of red wine (mainly polyphenols and especially resveratrol) since the so-called "French paradox" was first described [5] in order to explain the relationship observed between wine consumption and the incidence of cardiovascular disease (CVD). There is extensive evidence to support the influence of wine intake on cardiovascular health [6–9], controversy remains whether red wine in particular exerts beneficial effects compared with other alcoholic beverages [10,11] or simply alleviates the detrimental influence of alcohol on blood pressure (BP) [12,13]. No changes in BP were observed in

acute studies with healthy volunteers but an increase in the heart rate was reported after red wine consumption [14,15], whereas in coronary artery disease (CAD) patients, a decrease in systolic and diastolic BP was noted together with an increase in heart rate, 1 h post wine (red and white) intake [16]. Other studies have reported no changes in hemodynamics or BP after medium-term daily intake of either red wine or its dealcoholized equivalent [17,18].

Overall results of polyphenols consumption may be promising but they remain inconclusive and further prospective studies assessing dietary polyphenol exposure and studies using other methods to evaluate exposure (i.e., markers of consumption, metabolism, excretion) have been recommended, as concluded in a recent meta-analysis summarizing [19]. A recent systematic review using intervention studies confirmed that urinary polyphenol metabolites could serve as dietary biomarkers with high recovery yields and high correlations with intakes of polyphenol-rich food [20].

In this article, different grape pomace extracts were studied for their possible influence against hypertension disease, as they present an important under used residue of the wine making process. A large number of publications evidenced the abundance quantity of polyphenols in grape seeds and skins, showing significant antioxidant capacity. It is therefore obvious that it is a natural source of polyphenols that accounts for about 20% of grapes weight used to make wine [21,22]. Finally, some metabolites in urine, plasma and tissues were measured for accurate and precise estimation of dietary exposures and possible differences between the grape pomace extracts

2. Materials and Methods

2.1. Chemicals

Deionized water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). High Performance Liquid Chromatography (HPLC) grade acetonitrile (HPLC ≥ 99%), ethyl acetate (HPLC ≥ 99%), methanol (HPLC ≥ 99%), ethanol (HPLC ≥ 99%) and acetone purchased from Scharlau (Sentmenat, Barcelona, Spain). The following chemicals were obtained from Sigma Aldrich (Saint Louis, MO, USA): (+)-catechin (≥98%), (−)-epicatechin (≥98%), B_1 [(−)-epicatechin-(4β-8)-(+)catechin] (≥98%), procyanidin dimer B_2 [(−)-epicatechin-(4β-8)-(−)-epicatechin] (≥98%), cyanidin-3-*O*-glucoside chloride (≥98%), delphinidin-3-*O*-glucoside chloride (≥98%), malvidin-3-*O*-glucoside chloride (≥98%), peonidin-3-*O*-glucoside chloride (≥98%), gallic acid (≥98%), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8tetramethylchroman-2-carboxylic acid (Trolox) (≥97%), 2,2'-azinobis(3ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (≥98%), potassium persulfate (≥99%), fluorescein (≥98%), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) (≥97%), sodium dihydrogen phosphate dehydrate (≥ 98%), disodium hydrogen phosphate dodecahydrate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) (≥98%), iron (III) chloride hexa-hydrate (≥98%), iron (II) sulphate hepta-hydrate (≥98%), L-ascorbic acid (≥99%), derivatization reagent (pyridine (≥99.8%) and *N*-methyl-*N*(trimethylsilyl)trifluoroacetamide [MSTFA] (≥98.5%)) and *N*,*N*-dimethyl formamide (DMF) (≥99.8). The Laboratory of Organic Chemistry and Organometallic (Université Bordeaux 1) synthesized procyanidins dimers B_3 [(+)-catechin-(4α-8)-(+)-catechin] and B_4 [(+)-catechin-(4α-8)-(−)-epicatechin] and a trimer (T) [(+)-catechin-(4β-8)-(+)-catechin-(4β-8)-(−)-epicatechin] [23].

Ferulic acid (≥99%), was obtained from AASC Chemicals (Southampton, U.K.). EDTA and pentobarbital solution used in rats feeding studies were supplied by Sigma Aldrich. Hydrochloric acid (37%), sodium hydroxide (≥8%), acetic acid (≥99%), phosphoric acid (≥85%) and formic acid (≥95%), were purchased from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK).

2.2. Plant Materials

This study was conducted with 2010 grapes pomaces from *V. vinifera* L. cv. Grenache (from two different locations [GRE1 and GRE2]), Syrah (from two different locations

[SYR1 and SYR2]), Carignan (CAR), Mourvèdre (MOU), Counoise (COU) and Alicante (ALI), provided by the Château de Beaucastel located in the Vallée du Rhône, appellation of Châteauneuf-du-Pape. Details are provided below.

Samples were selected on the basis of their high content of polyphenols according to previous studies [24].

2.3. Animals for In Vivo Experiences

For in vivo experiments, spontaneously hypertensive rats (SHR) and normotensive control Wistar-Kyoto (WKY) rats were purchased from Laboratoire Janvier (Le Genest St. isle, France). Animals were maintained at a temperature of 23°C with 12 h light/dark cycles. Tap water and standard diet A 04 SAFE (Augy, France) composed of 83.9% of cereals and cereal by-products, 8.0% of vegetable proteins (soya bean meal, yeast), 4.1% of vitamin and mineral mixtures and 4% of animal proteins (fish) were given ad libitum. They were daily treated with grape pomace extract by gavage during 6 weeks at a dose of 21 mg/kg/day. Rats were divided in different groups: control group (6 WKY), SHR control group (5 SHR treated with 3% EtOH) and 6 groups of 4 SHR rats treated with pomace extract dissolved in 3% EtOH (SHR1: Grenache seed pomace extract, SHR2: Syrah seed pomace extract, SHR3: Syrah skin pomace extract, SHR4: Carignan seed pomace extract, SHR5: Mourvèdre skin pomace extract and SHR6: Alicante skin pomace extract). Blood pressure was measured by the tail-cuff method. The average of three pressure readings was recorded for each measurement.

Food intake and body weight of animals were recorded once a week. Prior to each experiment, adaptation period was allowed. Experiments were performed following European Community animal experiments ethical regulations.

The animal protocols used in this work were evaluated and approved by the Animal Use and Ethic Committee of Languedoc Roussillon Montpellier (reference CEEA-LR-13012) approved on 11 July 2013 and valid for 5 years. They are in accordance with European guidelines and French law for Laboratory Animal Experimentation.

2.4. Samples Preparation

2.4.1. Grape Pomace Samples Preparation

One hundred grams of GRE1, SYR1 and CAR seeds and GRE2, SYR1, SYR2, CAR, MOU and ALI skins were extracted in triplicate using 350 mL of distilled water for 1 h under magnetic agitation at 50 °C. In parallel, under the same conditions, these samples were also extracted using a 70% hydro-alcoholic solution (70:30, Ethanol:Water, v/v) solution. The centrifugal supernatants were evaporated and lyophilized to obtain two types of samples: an aqueous sample (EAQ) and a 70% hydro-alcoholic sample (EA70) for each variety and part.

2.4.2. Sample Collection from In Vivo Experiments

Urine was collected at two time points (0–8 h and 8–24 h) at day 1 and day 7 of gavage from metabolic cage in falcon tubes and immediately stored at −80 °C. At day 7, four hours after gavage, rats were anaesthetized with lethal dose of pentobarbital (60 g/L pentobarbital, 60 mg/kg body weight) and sacrificed by decapitation. Blood was drawn in an EDTA-moistened tube. Tubes were centrifuged at 2300× g for 10 min at 4 °C. Plasma was separated from erythrocytes before being stored at −80 °C. Tissues including heart, liver and kidneys were collected, rinsed, weighted, grounded to a powder and stored at −80 °C along with plasmas and urines prior to analysis.

2.5. Analytical Methods

2.5.1. Antioxidant Assays

The antioxidant capacity of pomace extracts was assessed by ABTS radical cation ($ABTS^{\bullet+}$), DPPH, Ferric Reducing Antioxidant Potential (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) test as it is described in previous studies [25].

2.5.2. Blood Pressure Measurement

Blood pressure was followed and accessed by tail-cuff method with a LETICA LE 5002 Scientific Instrument electrosphygomanometer (Panlab, Barcelona, Spain) under conscious condition, in a calmed and regulated between 29 °C and 32 °C darkened room. Rats were trained to the measurements, handled with care and covered with a fabric during the record in order to minimize stress. In this method, the reappearance of pulsation on a digital display of the blood pressure cuff is detected by a pressure transducer, amplified and recorded digitally as the systolic blood pressure. The average of the three pressure readings was recorded if only the difference between two measurements was below 20 mmHg.

2.5.3. Extraction of Phenolics from Tissues

Rat tissues from the same experimental group were pooled together in equa proportions. To 60 mg to freeze-dried tissues were added 50 µL of ascorbic acid 1% and 100 µL of phosphoric acid 4%. Each sample was spiked with 1 µg of ethyl gallate as an internal standard. The samples were first extracted with 800 µL of water/methanol/phosphoric acid 4% (94/4.5/1.5, *v*/*v*/*v*) using a sonicator for 30 s (Digital Sonifier® model S-150D ultrasonic cell disruptor, Branson, Teltow, Germany) and maintained in ice to avoid heat. Samples were then centrifuged for 15 min at 16,100× *g*, 20 °C in a 0.2 µm Micro-Spin™ Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, UK). The supernatant was decanted and the pellet re-extracted three more times with 500 µL of the same solvent as described above, after which it was centrifuged. The four supernatants were combined. A mixture of 1 mL of the assembled supernatant and 1 mL of phosphoric acid 4% was loaded onto OASIS® HLB cartridges (3cc, 60 mg) previously conditioned with 1 mL of methanol and 1 mL of 0.2% acetic acid. Column was washed with 1 mL of phosphoric acid 4% and with 1 mL of 0.2% acetic acid. The retained compounds were eluted with 2 × 1 mL acetone/Milli-Q water/acetic acid solution (70/29.5/0.5, *v*/*v*/*v*).

The eluate was reduced to dryness using a Speedvac concentrator (SPS SpeedVac, Thermo Savant, Waltham, MA, USA) and resuspended in 25 µL of acidified methanol (1% acid formic) to which was added 225 µL of 0.1% aqueous formic acid. Once resuspended, extracts were centrifuged at 16,100× *g* for 10 min at 4 °C in a 0.2 µm Micro-Spin™ Eppendorf filter (Alltech Associates Applied Sciences, Lancashire, UK) prior to analysis by HPLC-PDA-MS^n.

2.5.4. Extraction of Phenolics from Plasmas

Rat plasmas from the same group were pooled together in equal proportions before extraction [26]. Plasma samples were defrosted and spiked with 1 µg of ethyl gallate as an internal standard. Plasma was added drop wise while vortex to a 15 mL falcon tube containing 3.4% phosphoric acid and kept on a freeze water bath. Sample was then loaded onto OASIS® HLB cartridge (3 mL, 60 mg) previously conditioned with 1 mL of *N*,*N*-dimethyl formamide (DMF)/methanol (7/3, *v*/*v*) and 0.5% (*v*/*v*) acetic acid in water. Cartridge was washed with 3 mL of 0.5% acetic acid in water (*v*/*v*) and 1 mL of water/methanol/acetic acid (80/20/0.5). For elution, cartridge was dried and eluted with 2 × 1 mL of DMF/methanol (7/3, *v*/*v*). The eluate was collected in a tube containing 200 µL of 0.5% (*v*/*v*) acetic acid in methanol and reduced to approximately 50 µL using a Speedvac concentrator (SPS SpeedVac, Thermo Savant, Waltham, MA, USA). Sample was then resuspended in 25 µL of acidified methanol (1% acid formic) to which was added 225 µL of 0.1% aqueous formic acid. Extract was then centrifuged at 16,100× *g* for 10 min at 4 °C prior to analysis by HPLC-PDA-MS^n within 24 h.

2.5.5. Urine Analysis

Urine samples were defrosted, vortexed and centrifuged at 16,100× *g* for 10 min at 4°C prior to the analysis by HPLC-PDA-MS^n.

2.5.6. HPLC-PDA-ESI-MS^n Analysis of Procyanidin Metabolites

Quantification of metabolites in urines, plasma and tissues was carried out using two Surveyor HPLC systems, both equipped with sampler cooler maintained at 4 °C and a PDA detector. One of them was equipped with a Finnigan LCQ Duo ion trap mass spectrometer for urine analysis. The second one equipped with a LCQ Advantage ion trap mass spectrometer for tissue and plasma analysis.

Mass spectrometers were both fitted with an electrospray interface (ESI) (Thermo Fisher scientific, San Jose, CA, USA). Separation was performed on a 250 × 4.6 mm i.d. 5 µm Kinetex phenyl-hexyl 100 Å column (Phenomenex, Macclesfield, U.K.), maintained at 40 °C. The mobile phase was pumped at a flow rate of 1 mL/min with a gradient over 30 min of 5–60% methanol in 0.1% aqueous formic acid (analysis of urines) or a gradient over 30 min of 5–65% in 0.1% aqueous formic acid (analysis of tissues). The column eluate initially passed through the PDA detector and was then split, with 0.2 mL/min directed to the mass spectrometer fitted with an electrospray interface operating in negative ion mode. The tuning of the mass spectrometer was optimized by infusing a standard of (−)-epicatechin, dissolved in the initial HPLC mobile phase, into the source at a flow rate of 0.2 mL/min.

Procyanidin metabolites were firstly identified using full-scan data-dependent MS^2 scanning from m/z 100 to 700. Compound identities were confirmed by MS^3 consecutive reaction monitoring with a collision energy set at 30%. Following HPLC separation and MS^3 identification, flavan-3-ols and their metabolites were quantified using selective reaction monitoring (SRM mode). Quantification of 5-(hydroxyphenyl)-4-hydroxyvaleric acid-*O*-glucuronide (m/z, 401/225) and 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate (m/z, 287/207) was by reference to the aglycone 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone while 5-(hydroxyphenyl)-4hydroxyvaleric acid-*O*-sulphate (m/z, 305/225) levels were measured using a calibration curve obtained with 5-(3′,4′-dihydroxyphenyl)-4-hydroxyvaleric acid.

The other metabolites were quantified as (−)-epicatechin equivalents.

2.5.7. Statistical Analyses

All measurements were performed in triplicate. Results are expressed as means ± standard deviation (SD). One-way ANOVA was performed to test the effects of variation factors (different samples) on each variable. If significant effects were found at a 95% confidence interval, ANOVA was followed by a Tukey's HSD and Duncan post hoc test to identify differences among groups. These analyses were performed using Statistica V.7 Software (Statsoft Inc., Tulsa, OK, USA).

3. Results and Discussion

3.1. Grape Pomace Seed and Skin Antioxidant Activities Evaluation

The antioxidant potential was determined in order to select the most active grape pomace seeds and skins among the studied varieties. The choice of assay method is often based on speed, simplicity, ease of use and instrumentation availability. More than one type of measurement needs to be performed to take into account the various mode of action of antioxidants [27,28]. In this work, the free radical scavenging potential was evaluated by three spectrophotometric tests: the FRAP, $ABTS^{\bullet+}$ and DPPH and a spectrofluorometric test, the ORAC test.

Concerning seed extracts, the four antioxidant analytical techniques gave the same classification both for EAQ and EA70. The highest antioxidant activities were found in SYR1 for both types of extracts. Results were correlated with previous analyses which evidenced SYR1 as having a substantial amount of flavan-3-ols, procyanidins and anthocyanins. Antioxidant activities of EAQ and EA70 grape pomace seed extracts were shown in Table 1.

Table 1. Antioxidant activity characterization in EAQ and EA70 grape pomace seed extracts.

		ORAC [2]	FRAP [2]	ABTS$^{\bullet+}$ [2]	DPPH [2]
Seeds-EAQ	**GRE1** [1]	1466.4 ± 29.6 [a]	0.63 ± 0.02 [a]	1203.2 ± 24.1 [a]	410.8 ± 43.3 [a]
	SYR1 [1]	2230.7 ± 101.7 [b]	1.33 ± 0.08 [c]	2432.6 ± 56.0 [c]	1037.1 ± 64.0 [b]
	CAR [1]	2058.6 ± 85.1 [b]	1.06 ± 0.08 [b]	1948.8 ± 61.1 [b]	1050.6 ± 30.1 [b]
Seeds-EA70	**GRE1** [1]	1926.7 ± 108.6 [a]	1.28 ± 0.01 [a]	2813.2 ± 90.0 [a]	1277.6 ± 54.7 [a]
	SYR1 [1]	2614.0 ± 150.9 [a]	1.45 ± 0.16 [a]	3601.2 ± 88.6 [b]	1685.9 ± 130.7 [b]
	CAR [1]	2332.9 ± 91.9 [a]	1.20 ± 0.06 [a]	3495.6 ± 66.4 [b]	1536.8 ± 38.9 [b]

[1] GRE1, Grenache; SYR1, Syrah; CAR, Carignan. Data are expressed as the mean of triplicate ± SD. [2] ORAC, ABTS$^{\bullet+}$ and DPPH are expressed as µmol Trolox/g Dry Weight (DW) and FRAP as mmol Fe^{2+}/g DW. [a,b,c]; Anova was performed to compare values obtain between varieties for the same test. Same letters indicate no significant differences between the value (Tukey's test, $p < 0.05$).

In skins, results obtained by the different antioxidant analyses were more disparate, especially in EA70 extracts (Table 2). In aqueous extracts, the highest antioxidant activity was found in SYR1 and ALI. This observation was observed with every test and correlated well with previous results evidencing these extracts as containing high phenolic content. In EA70, different antioxidant tests did not give the same extract classification. Despite this fact, SYR1 skin extract was classified as being the first or second extract showing the highest antioxidant capacity in the four tests (ORAC: 1912.6 µM TE/g DW; FRAP: 1.52 mM Fe^{2+}/g DW, ABTS$^{\bullet+}$: 2614.5 µM TE/g DW and DPPH: 1391.7 TE/g DW).

Table 2. Antioxidant activity characterisation in EAQ and EA70 grape pomace skin extracts.

		ORAC [2]	FRAP [2]	ABTS$^{\bullet+}$ [2]	DPPH [2]
Skins-EAQ	**GRE2** [1]	1190.7 ± 183.6 [ab]	0.56 ± 0.01 [c]	934.1 ± 11.9 [b]	99.5 ± 10.8 [a]
	SYR1 [1]	1345.9 ± 19.2 [ab]	0.88 ± 0.01 [e]	1428,0 ± 54.8 [c]	690.3 ± 147.0 [bc]
	SYR2 [1]	1066,0 ± 84.2 [a]	0.14 ± 0.02 [a]	668.3 ± 30.0 [a]	263.9 ± 71.5 [ab]
	CAR [1]	1077.8 ± 60.2 [a]	0.67 ± 0.02 [d]	1048.8 ± 101.6 [b]	591.0 ± 85.6 [abc]
	MOU [1]	1033.8 ± 77.6 [a]	0.32 ± 0.01 [b]	965.6 ± 16.6 [b]	279.4 ± 61.7 [ab]
	ALI [1]	1714.6 ± 14.8 [b]	1.13 ± 0.00 [f]	1760.1 ± 91.0 [d]	1057.1 ± 45.2 [c]
Skins-EA70	**GRE2** [1]	1828.3 ± 40.4 [bc]	1.32 ± 0.03 [c]	2612.1 ± 130.9 [a]	877,0 ± 74.3 [a]
	SYR1 [1]	1912.6 ± 6.1 [bc]	1.52 ± 0.05 [d]	2614.5 ± 10.4 [a]	1391.7 ± 37.2 [bc]
	SYR2 [1]	1701.8 ± 88.3 [bc]	0.94 ± 0.03 [a]	2010.6 ± 147.0 [a]	1164.9 ± 55.6 [ab]
	CAR [1]	1238.4 ± 11.1 [a]	1.34 ± 0.03 [c]	2555.9 ± 146.0 [a]	1075.4 ± 46.2 [ab]
	MOU [1]	2070.0 ± 60.6 [c]	1.03 ± 0.02 [ab]	2674.8 ± 187.3 [a]	833.3 ± 26.4 [a]
	ALI [1]	1628.5 ± 82.6 [b]	1.13 ± 0.01 [b]	1923.4 ± 87.0 [a]	1749.3 ± 112.7 [c]

[1] GRE2, Grenache; SYR1 and SYR2, Syrah; CAR, Carignan; MOU, Mourvèdre; ALI, Alicante. Data are expressed as the mean of triplicate ± SD. [2] ORAC, ABTS$^{\bullet+}$ and DPPH are expressed as µmol Trolox/g DW and FRAP as mmol Fe^{2+}/g DW. [a,b,c,d,e,f]; Anova was made to compare values obtain between varieties for the same compound. Same letters indicate no significant differences between the value (Tukey's test, $p < 0.05$).

EA70 extracts exhibited higher potential and proved to be more effective than EAQ extracts. Because of bioavailability, metabolism, biotransformation and chemical reactivity, in vitro capacity cannot be simply extrapolated [29]. Therefore, in order to evaluate the health effects of these extracts, in vivo experiments need to be performed and the effects of antioxidant may be evaluated using appropriate biomarkers in biological fluids and tissues.

3.2. In Vivo Results

3.2.1. Systolic Blood Pressure Results

Spontaneously hypertensive rats (SHR) selected for this study is frequently used to carry out studies on the antihypertensive effect of functional food ingredients. This strain represents nowadays the best experimental model for essential hypertension in humans and has shown its efficiency in many studies [30–35].

In order to evaluate the in vivo effect of grape pomace extracts and their potential effect on hypertension, rats were fed with different grape pomace EA70 extracts at a dose of 21 mg/kg/day, equivalent to a daily dose of 70 kg human consumption of 0.5 L of wine. The study was conducted over six weeks including three weeks of treatments, one week of treatment resumption followed again by two weeks of treatment.

The mean systolic blood pressure of SHR rats was comprised between150 mmHg at the beginning of the experiment and 190 mmHg after five weeks (Figure 1).

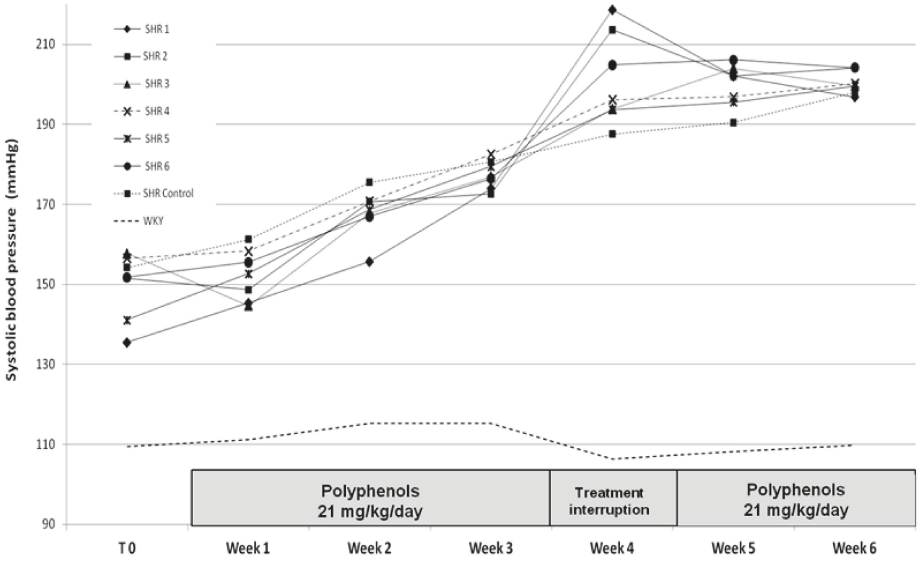

Figure 1. Effect of polyphenolic extracts on the mean systolic blood pressure during the 6-week study.

Polyphenolic extracts given to SHR rats seemed to have little effect on systolic blood pressure which increased gradually (except for the SHR1 after 1 week of treatments). However, after three weeks of extracts intake, gavage intolerance was observed and caused difficulty with the administration of polyphenolic extracts, forcing the interruption of the treatment for one week. This treatment interruption was followed by an increase of the systolic blood pressure in SHR1 (Grenache seed pomace extract), SHR2 (Syrah seed pomace extract) and SHR6 (Alicante skin pomace extract) group compared to the SHR control group. This phenomenon can be interpreted as a « rebound effect » commonly observed with anti-hypertensive drugs and may reveal an antihypertensive effect of grape pomace extracts. The treatment resumption at weeks 5 and 6 describe a relative decrease in systolic blood pressure for SHR1 and SHR2 groups and a stabilisation of systolic blood pressure for the SHR6 group, which implies that if the treatment was pursued for a higher period of time, the values of systolic blood pressure may have been improved significantly with respect to the SHR control group.

3.2.2. Quantification of Polyphenolic Metabolites in Urine

Based on previous results, extracts SHR1 (GRE1 (EA70) seed pomace extract), SHR5 (MOU (EA70) skin pomace extract) and SHR6 (ALI (EA70) skin pomace extract) have been chosen to bioavailability study.

Urine samples were collected at 0–8 h and 8–24 h on day 1 and day 7 after the ingestion of different grape pomace extracts. The urine did not contain any of the original grape pomace extract flavan-3-ols and procyanidins but glucuronides and methylglucuronides of (epi)catechin, as well as valerolactone

and valeric acid phase II glucuronide and sulphate metabolites were detected. Compared to other studies in which rats were fed grape derived products [36–38], sulphate derivatives were found as one of the main metabolites, whereas in this work, sulphates were present in very low amounts. The reason for these varying metabolite profiles, especially the dominance of glucuronides in some studies and sulphates in order could be due to losses of (epi)catechin-*O*-sulphates during sample processing before analysis [39]. The difference in the metabolic profile of (epi)catechins may also be attributed to the different flavan-3-ols composition of the matrix (e.g., grape seed/skin extracts, tea, cocoa).

A total of 18 metabolites were identified in urine. To have an overall picture of metabolite excretion, in this article, the sum of urinary (epi)catechin-*O*-glucuronide, *O*-methyl-(epi)catechin-*O*-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide, 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate, 5hydroxyphenyl-4-hydroxyvaleric acid-*O*-sulphate, 5-(phenyl)-4-hydroxyvaleric acid-*O*sulfate, isoferulic acid-4-*O*-sulphate was calculated. The results are presented in Tables 3 and 4.

Table 3. Sum of structurally related urinary metabolites at day 1, 0–8 h and 8–24 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SD.

Σof Structurally Related Metabolites(Day 1, 0–8 h)	**SHR1**	**SHR5**	**SHR6**	**Control**
(Epi)catechin-*O*-glucuronide	**1.0 ± 0.3** *	**2.0 ± 0.2** *	**1.2 ± 0.5** *	0.1. ± 0.0
O-Methyl-(epi)catechin-*O*-glucuronide	**8.5 ± 1.5** *	3.5 ± 0.4	6.1 ± 2.7	3.5 ± 1.0
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**1.0 ± 0.1** *	1.2 ± 0.4	0.5 ± 0.3	0.4 ± 0.2
5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	1.5 ± 0.3	4.4 ± 2.9	1.2 ± 0.4	1.1 ± 0.3
5-(Hydroxyphenyl)-γ-hydroxyvaleric acid-*O*-sulphate	**2.5 ± 0.6** *	3.5 ± 1.3	**2.0 ± 0.5** *	0.9 ± 0.3
5-(Phenyl)-γ-hydroxyvaleric acid-*O*-sulphate	**6.9 ± 1.3** *	7.6 ± 1.6	6.1 ± 3.7	3.1 ± 0.7
Isoferulic acid-4-*O*-sulfate	**157.0 ± 35.5** *	**75.0 ± 15.6***	82.8 ± 19.9	82.2 ± 19.9
Σof Structurally Related Metabolites(Day 1, 8–24 h)	**SHR1**	**SHR5**	**SHR6**	**Control**
(Epi)catechin-*O*-glucuronide	1.1 ± 0.2	1.8 ± 0.6	1.4 ± 0.3	1.0 ± 0.3
O-Methyl-(epi)catechin-*O*-glucuronide	3.8 ± 0.7	**6.9 ± 2.1** *	**7.1 ± 1.3** *	2.7 ± 1.0
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.2 ± 0.0	0.4 ± 0.1	**0.4 ± 0.1** *	0.2 ± 0.0
5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	1.3 ± 0.2	1.8 ± 0.6	1.6 ± 0.2	1.3 ± 0.3
5-(Hydroxyphenyl)-γ-hydroxyvaleric acid-*O*-sulphate	1.0 ± 0.1	**2.6 ± 0.9** *	**2.2 ± 0.4** *	0.7 ± 0.1
5-(Phenyl)-γ-hydroxyvaleric acid-*O*-sulphate	12.7 ± 1.8	14.4 ± 2.5	**15.8 ± 1.1** *	8.7 ± 0.9
Isoferulic acid-4-*O*-sulfate	80.4 ± 13.6	99.7 ± 16.1	**111.5 ± 25.7** *	74.2 ± 4.3

* Metabolites that were excreted in significantly higher amounts compared to their respective control ($p < 0.05$). SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract.

Table 4. Sum of structurally related urinary metabolites at day 7, 0–8 h and 8–24 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SD.

Σof Structurally Related Metabolites(Day 7, 0–8 h)	**SHR1**	**SHR5**	**SHR6**	**Control**
(Epi)catechin-*O*-glucuronide	1.6 ± 1.0	3.1 ± 1.9	**2.9 ± 1.0** *	0.5 ± 0.3
O-Methyl-(epi)catechin-*O*-glucuronide	5.7 ± 2.2	5.7 ± 2.3	**5.9 ± 2.1** *	1.7 ± 0.4
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	0.2 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	0.2 ± 0.1
5-(Hydroxyphenyl)-γ-hydroxyvaleric acid-*O*-sulphate	0.4 ± 0.1	**0.6 ± 0.1** *	0.9 ± 0.4	0.4 ± 0.2
5-(Phenyl)-γ-hydroxyvaleric acid-*O*-sulphate	6.6 ± 1.6	6.7 ± 2.6	8.4 ± 2.3	7.7 ± 2.0
Isoferulic acid-4-*O*-sulfate	**131.2 ± 25.9** *	**149.2 ± 34.0** *	137.4 ± 45.8	71.8 ± 29.5
Σof Structurally Related Metabolites(Day 7, 8–24 h)	**SHR1**	**SHR5**	**SHR6**	**Control**
(Epi)catechin-*O*-glucuronide	1.6 ± 0.4	1.6 ± 0.1	**2.2 ± 0.4** *	1.1 ± 0.3
O-Methyl-(epi)catechin-*O*-glucuronide	8.1 ± 1.8	6.2 ± 1.3	**5.2 ± 0.8** *	3.7 ± 0.8
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	0.5 ± 0.1	0.7 ± 0.3	**0.7 ± 0.1** *	0.4 ± 0.1
5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	0.8 ± 0.1	1.4 ± 0.5	1.6 ± 0.3	1.1 ± 0.3
5-(Hydroxyphenyl)-γ-hydroxyvaleric acid-*O*-sulphate	1.2 ± 0.4	1.8 ± 0.6	1.4 ± 0.3	1.1 ± 0.3
5-(Phenyl)-γ-hydroxyvaleric acid-*O*-sulphate	13.2 ± 4.2	15.4 ± 3.6	14.5 ± 4.0	12.4 ± 2.8
Isoferulic acid-4-*O*-sulfate	**209.4 ± 38.6** *	**218.2 ± 60.6** *	171.5 ± 43.0	121.2 ± 20.8

* Metabolites that were excreted in significantly higher amounts compared to their respective control ($p < 0.05$). SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract.

Higher levels of *O*-methyl-(epi)catechin-*O*-glucuronide relative to (epi)catechin-*O*-glucuronide were found in every urine sample. Sulphate derivatives of 5-(hydroxyphenyl)-γ-valerolactone were excreted in greater amounts than glucuronides. A decrease of 5-(hydroxyphenyl)-4-hydroxyvaleric

acid-*O*-sulfate from 0–8 h to 8–24 h at day 1 was observed while an increase of 5-(phenyl)-4hydroxyvaleric acid-*O*-sulphate occurred at 8–24 h compared to 0–8 h in all rat urines at day 1 and day 7. A previous report on a human almond skin polyphenol bioavailability [40] suggested that partial dihydroxylation reactions occur gradually as colonic metabolism progresses. These authors observed a change in the hydroxylation pattern of the phenyl ring from di- to mono- and unhydroxylated forms. Dihydroxylated derivatives were found 6–10 h after the intake, monohydroxylated forms were observed at 6–24 h and unhydroxylated derivatives were found 10–24 h after the intake.

In order to compare efficiency of grape pomace extracts, the sum of metabolites that are excreted in significantly higher amounts compared to their respective control ($p < 0.05$) are presented in Figure 2 for days 1 and 7.

At day 1 of SHR rat gavage, substantial 0–8 h urinary excretion of metabolites was observed in the SHR1 and SHR5 experimental groups unlike SHR6 in which highest excretion occurred in 8–24 h urine. Considering the 0–8 h samples, high urinary excretion was found in E5 (80 ± 16 nmol) followed by E1 (70 ± 16 nmol). During 8–24 h collection period, excretion of metabolites was more pronounced with E6 (80 ± 7 nmol).

At day 7 no significant difference was observed between different experimental groups in both 0–8 h, 8–24 h and even over 0–24 h. This phenomenon can be due to a high absorption and excretion reducing variability between subjects of the same group. Overall, total 24 h urinary excretion at day 7 ranged from 117 ± 33 nmol in SHR6 to 160 ± 40 nmol in SHR5 and was higher than that obtained at day 1 which varied from 109 ± 29 to 127 ± 17 nmol.

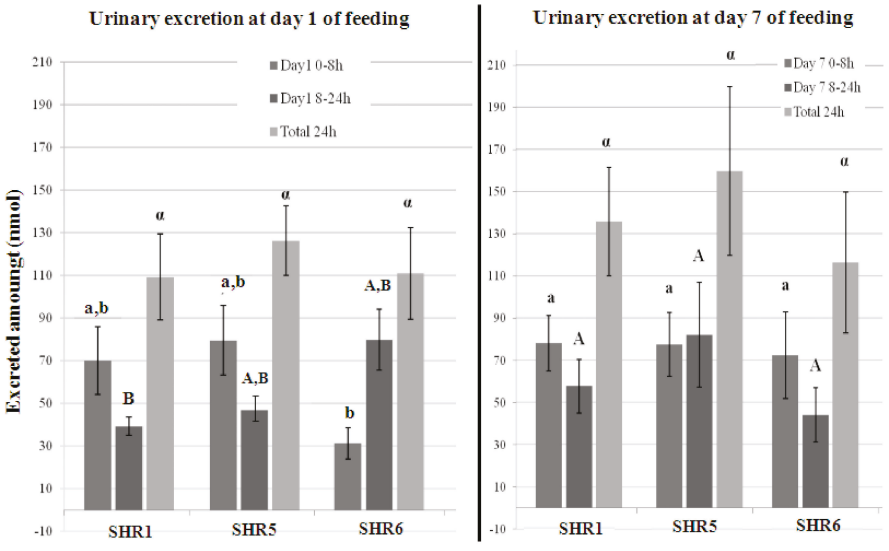

Figure 2. Total urinary metabolites excreted at days 1 and 7, 0–8 h, 8–24 h and 0–24 h after ingestion of grape pomace extracts. SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract. a,A,α,B,b: Anova was performed to compare values between different experimental groups during 0–8 h (small letters), 8–24 h (capital letters) and 24 h (symbol letters) at day 1. Same letters indicate no significant differences between the values ($p < 0.05$). Data are expressed as mean values in nmol ± standard error.

The percentage of intake of grape pomace extracts administrated alone or in association with verapamil to SHR rats was calculated for each time point (Table 5).

Table 5. Percentage of intake of different grape pomace extracts excreted in urine at day 1 and day 7, 0–8 h, 8–24 h and 0–24 h after intake.

	Percentage of Intake Day 1 (%)			Percentage of Intake Day 7 (%)		
	SHR1 [a]	**SHR5** [a]	**SHR6** [a]	**SHR1** [a]	**SHR5** [a]	**SHR6** [a]
0–8 h	0.83	0.83	0.51	0.94	2.10	1.03
8–24 h	0.19	0.74	0.51	0.58	2.31	0.39
Total 24 h	1.02	1.57	1.02	1.52	4.41	1.43

[a] SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract.

At day 1, excretion as a percentage of intake varied from 1.02% to 1.57%. Highest recoveries were observed in the SHR5 group (1.57%, over 0–24 h). At day 7, an increase of intake was observed for the two collection periods and, as a consequence, over 0–24 h. The recoveries ranged from 1.43% in SHR6 to 4.41% in SHR5. A large increase in metabolite excretion was observed in SHR5 group with 4.41% of intake at day 7 compared to 1.57% at day 1. These observations suggested that over time, SHR rats may be able to ingest higher doses of polyphenols especially those contained in MOU (EA70) skin pomace extract.

The recoveries obtained in this study were low compared to other studies. For instance, a urinary excretion of intake 0–24 h corresponding to 33% was found after ingestion of a grape seed extract containing a wide array of monomeric to polymeric flavan-3-ols ingestion [36]. In humans, urinary recovery of flavan-3-ols accounted 2–10% for red wine catechins [41]. However, higher amounts of flavan-3-ol monomers and polymers were ingested by humans than rats. A research [42] showed in a green tea flavan-3-ol feeding study with ileostomists that (epi)gallocatechins are subjected to strictly limited absorption whereas (epi)catechins can be readily absorbed even with an increasing dose. Thus, the low number of polyphenols fed in this study compared to other studies could be the cause of these recovery differences.

3.2.3. HPLC-ESI-MS^n Analysis of SHR Rat Plasma

SHR rats' plasma was screened for flavan-3-ol and procyanidin metabolites using, in the first instance, the full-scan MS mode from m/z 100 to 700 in negative ionization mode. Peak identifications were based on data dependent MS^2 and on previous studies [36,37,43,44].

A total of 11 metabolites were found in SHR rat plasma collected 4 h after grape pomace extract ingestion. Plasma did not contain any of the original grape pomace extract flavan-3-ols and procyanidins but glucuronide, methyl-glucuronide and di-methylglucuronide of (epi)catechin and glucuronide, sulphate and mono-substituted valerolactone and valeric acid were present. These metabolites were also detected in urine with the exception of the di-methyl-(epi)catechin-*O*-glucuronide. Metabolites were occurred mainly as glucuronides rather than sulphates. These observations were in accordance with a previous study by [36] in which rats were fed with grape seed extracts. These investigators detected only glucuronidated forms of (epi)catechins in plasma while sulphates predominated in urine.

Quantitative data are presented in Table 6. Generally, lower amounts of metabolites were observed in plasma than in urine showing that flavan-3-ols are rapidly turned over the circulatory system, and, rather than accumulating, are excreted via the kidneys. Figure 3, summarizes plasma metabolite amounts in different experimental group at day 7, 4 h after grape pomace extracts ingestion ($p < 0.05$) and their respective recoveries (%).

Table 6. Mean plasma metabolite levels at day 7, 4 h after ingestion of grape pomace extracts. Data are expressed as nmol ± SD.

Metabolites	SHR1	SHR5	SHR6
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 1	**0.62 ± 0.06 ***	0.71 ± 0.02	0.46 ± 0.01
(Epi)catechin-*O*-glucuronide 1	Nd	Nd	**0.25 ± 0.02 ***
(Epi)catechin-*O*-glucuronide 2	**0.34 ± 0.01 ***	0.32 ± 0.03	**0.39 ± 0.03 ***
O-Methyl-(epi)catechin-*O*-glucuronide 1	**0.24 ± 0.02 ***	**0.17 ± 0.02 ***	**0.23 ± 0.01 ***
5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide 2	**0.26 ± 0.01 ***	**0.31 ± 0.00 ***	**0.22 ± 0.01 ***
Methyl-(epi)catechin-*O*-glucuronide 2	**0.62 ± 0.05 ***	0.41 ± 0.00	**0.71 ± 0.04 ***
Methyl-(epi)catechin-*O*-glucuronide 3	Nd	Nd	**0.37 ± 0.01 ***
Di-methyl-(epi)catechin-*O*-glucuronide 1	**0.54 ± 0.06 ***	**0.86 ± 0.03 ***	**0.50 ± 0.02 ***
5-(Hydroxyphenyl)-4-hydroxyvaleric acid-*O*-sulphate 2	Nd	Nd	Nd
Di-methyl-(epi)catechin-*O*-glucuronide 2	**0.42 ± 0.04 ***	**0.35 ± 0.04 ***	**0.19 ± 0.01 ***
5-(Hydroxyphenyl)-γ-valerolactone sulphate 1	**0.42 ± 0.04 ***	**0.31 ± 0.05 ***	**0.42 ± 0.06 ***

* Metabolites that were excreted in significantly higher amounts compared to their respective control ($p < 0.05$). SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract. SD, standard deviation. Nd, not detected.

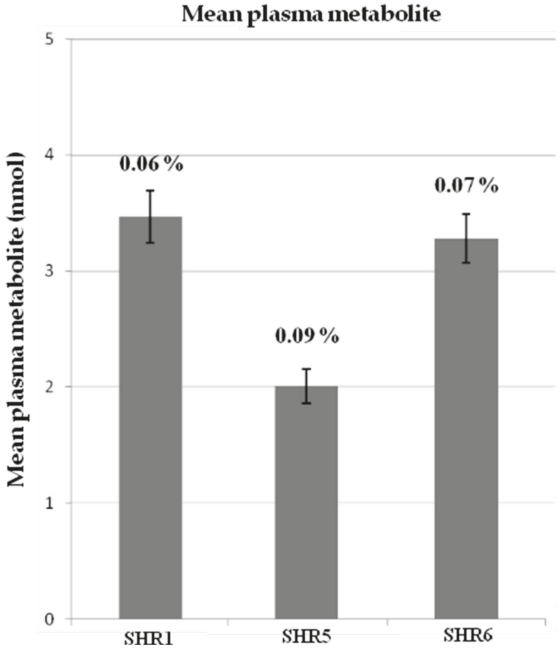

Figure 3. Mean plasma metabolite levels in different experimental group at day 7, 4 h after ingestion of grape pomace extracts ($p < 0.05$) and their respective recoveries (%). Data expressed as nmol ± standard deviation. SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract.

SHR5 urinary excretion indicated substantial absorption of polyphenols (4.41% of intake) but a recovery of 0.09% in plasma implies a rapid turnover of polyphenols in the circulatory system. Grape omace polyphenols were absorbed to a lesser extent by rats fed with extracts of SHR1 and SHR6, as illustrated by low recoveries of metabolites.

3.2.4. HPLC-ESI-MS^n Analysis of SHR Rat Tissues (Heart, Liver and Kidneys)

Metabolites in SHR rat tissues were detected using the same procedure as described for urine and plasma. No (epi)catechin conjugates were found but a 5-(hydroxyphenyl)-γ-valerolactone-*O*-glucuronide (*m*/*z* 383/207, 163) and a 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate (*m*/*z* 287/207, 163) were detected and quantified by SRM. Their distribution in tissues is presented in Table 7. Data are expressed as nmol per organ ± standard deviation. Both glucuronide and sulphate metabolites of the 5-(hydroxyphenyl)-γ-valerolactone were detected but the glucuronide predominated.

Heart tissues contained more glucuronidated metabolites than sulphated. 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate, was detected only in E1 and E5 experimental groups at a level of 0.11 ± 0.00 nmol.

Kidneys contained more 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate than the liver.

Liver contained the highest level of metabolites with most present as glucuronides. A 5-(hydroxyphenyl)-γ-valerolactone-*O*-sulphate was only detected in SHR rats fed with ALI (EA70) skin pomace extract + verapamil at 7.24 ± 0.6 nmol. Only SHR from E5, VE5 and VE6 experimental groups contained significantly higher amounts (210 ± 12 nmol, 198 ± 3 nmol and 194 ± 7 nmol, respectively) of 5-(hydroxyphenyl)-γ-valerolactone-*O*glucuronide compared to control groups.

Table 7. Mean tissue metabolite levels at day 7, after 4 h of grape pomace extracts ingestion. Data are expressed as nmol per organ ± standard deviation.

	Mean Tissue Metabolites Levels (Day 7, after 4 h)	SHR1	SHR5	SHR6
Heart	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	**2.50 ± 0.25 ***	**2.64 ± 0.03 ***	**2.27 ± 0.08 ***
	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	0.11 ± 0.00 *	**0.11 ± 0.00 ***	Nd
Kidneys	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	5.55 ± 0.11	6.62 ± 0.08	5.23 ± 0.34
	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	**0.38 ± 0.00 ***	**0.37 ± 0.02 ***	**0.45 ± 0.03 ***
Liver	5-(Hydroxyphenyl)-γ-valerolactone-*O*-glucuronide	167.19 ± 8.77	**209.98 ± 12.20 ***	179.26 ± 0.25
	5-(Hydroxyphenyl)-γ-valerolactone-*O*-sulphate	Nd	Nd	Nd

* Metabolites that were excreted in significantly higher amounts compared to their respective control ($p < 0.05$). SHR rats fed with: SHR1, Grenache (GRE1) EA70 seed pomace extract; SHR5, Mourvèdre (MOU) EA70 skin pomace extract and SHR6, Alicante (ALI) EA70 skin pomace extract. SD, standard deviation. Nd, not detected.

The absorption and arguably metabolism, of flavan-3-ols and procyanidins initially takes place during transfer through the wall of the small intestine after which further phase II metabolism occurs in the liver. As liver appear to be the most important organs involved in flavonoid metabolism, it was not surprising to find such a high level of metabolites. Some of the conjugated metabolites can be actively effluxed back into the lumen of the small intestinal and/or may be transported to other organs through the bloodstream as shown by their presence in the heart. In addition, metabolites were also detected in kidneys no doubt as a consequence of renal excretion. It should be noted that the time of tissue sampling may be of importance and metabolites detection depends on the kinetics of their accumulation and elimination in the tissues. In this study, tissues which were shown to contain metabolites were collected 4 h after the ingestion of grape pomace extracts. A study carried out in 2005 found flavan-3-ol metabolites in the liver of rats 1 h and 4 h after ingestion of a grape seed extract but none were detected 6, 12 and 24 h after intake [36]. This indicates a rapid elimination of flavan-3-ol metabolites in keeping with them being treated as xenobiotics by the body.

4. Conclusions

This study confirms that substantial levels of polyphenols after the winemaking process, remain in pomace in quantities sufficient to exert anti-hypertensive effects. In addition, according to the extract used and its composition, it is feasible to modulate anti-hypertensive effects by amplifying or decreasing polyphenols absorption. Therefore, it will be interesting to elucidate the exact mechanisms and compounds involved in this phenomenon in order to have a better control on blood pressure regulation and facilitate the choice of effective grape pomace extracts for

further experiments. Moreover, it will be useful to investigate the effect of different flavan-3-ol fractions (i.e., oligomeric, monomeric) and anthocyanin fractions (i.e., glucosides, acetylated glucosides and coumarylic glucosides) in order to identify whether anti-hypertensive effects are linked to a particular compound or to the extracts as a whole.

As SHR represents a good model to investigate hypertension, studies could be extended to human clinical trials. For clinical tests, different parameters have to be taken into account such as the subjects (i.e., pre-hypertensive or hypertensive subjects), the dose used, the diet, biological fluid collections and biological markers to be quantified. In addition, different processes will have to be considered such as the election of grape pomace varieties and their parts (seeds/skins), the extraction processes which will be used, the dosage and the galenic formulation used in order to provide great stabilisation of the active substances.

Moreover, analyses on SHR faeces must be done to complete the polyphenols excretion.

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diseases

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Review

Potential Beneficial Effects of Wine Flavonoids on Allergic Diseases

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Abstract: Wine, a widely consumed beverage, comprises several biophenols that promote health. Flavonoids, majorly present in red wine, have been shown to have antioxidant, anti-inflammatory, anticancer, and immunomodulatory activities. Regular consumption of red wine (100 mL/day) is estimated to provide an average of 88 mg of flavonoids, whereas recent epidemiological studies indicate that wine is one of the major sources of flavonoid intake amongst wine lovers in European countries (providing an average intake of 291–374 mg/day of flavonoids). In addition to being antioxidants, in vitro studies suggest that flavonoids also have anti-allergic activities that inhibit IgE synthesis, activation of mast cells and basophils or other inflammatory cells, and production of inflammatory mediators, including cytokines. Furthermore, they affect the differentiation of naïve CD4+ T cells into effector T cell subsets. Moreover, several studies have reported the benefits of flavonoids in allergic models such as atopic dermatitis, asthma, anaphylaxis, and food allergy; however, evidence in humans is limited to allergic rhinitis and respiratory allergy. Although further evaluation is required, it is expected that an appropriate intake of flavonoids may be beneficial in preventing, and eventually managing, allergic diseases.

Keywords: allergy; antioxidant; wine flavonoids

1. Introduction

The prevalence and incidence of allergic diseases, such as allergic rhinitis, asthma, atopic dermatitis, and food allergy, have increased worldwide during the past two to three decades [1,2]. The environmental and genetic interaction leads to sensitivity in individuals towards environmental allergens, then causes allergic diseases [3–5]. The "diet hypothesis" proposes that changes in dietary habit may play a significant role in the increase, since foods and beverages contain allergy-promoting and anti-allergic nutrients [6–8]. Minerals such as selenium, copper, zinc, and magnesium, vitamins A, C, D, and E, probiotics, and omega-3 polyunsaturated fatty acids (PUFAs) possess anti-allergic functions, whereas omega-6 PUFAs are precursors for leukotriene C4, which promotes allergic inflammation [6].

Flavonoids, polyphenolic plant secondary metabolites, have antioxidant, anti-inflammatory, and anti-allergic activities as well as immunomodulating effects [9,10]. Red wine, a major source of flavonoids for wine lovers, is known to reduce cardiovascular events when consumed in moderation [11]. Although the benefits of red wine in allergic diseases have not been elucidated in detail, based on recent findings, the present article emphasizes that an appropriate intake of flavonoids may be beneficial in preventing, and eventually managing, allergic diseases.

2. Flavonoids, the Major Ingredient in Red Wine for Promoting Health

Flavonoids are found in fruits, vegetables and tea, thus forming common ingredients of the daily diet [12–14]. Flavonoids, which share a common structure comprising two aromatic rings (A and B) bound together by three carbon atoms forming an oxygenated heterocycle (ring C) (Figure 1), are generally classified into six subclasses: flavones (luteolin, apigenin, and baicalein), flavonols (fisetin, kaempferol, quercetin, myricetin, and isohamnetin), flavanones (hesperetin, naringenin, and eriodictyol), isoflavones (daidzein and genistein), anthocyanidins (cyanidin, delphinidin, malvidin, pelargonidin, petunidin, and peonidin) and flavanols (catechins and proanthocyanidins).

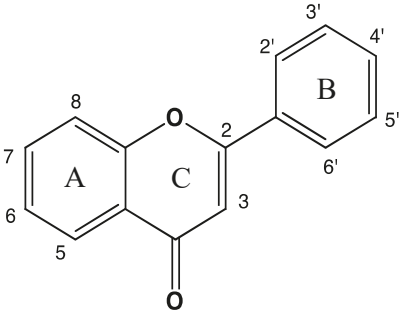

Figure 1. Structure of basic flavonoid skeletons.

Wine is a dietary source of phenolic compounds, namely flavonoids and non-flavonoids, which include phenolic acids, phenols, and stilbenes [11]. Recent developments regarding the flavonoid content of foods and beverages in the databases of the US Department of Agriculture (USDA) [15], the European BioActive Substances in Food Informative System (EuroFIR-BASIS) [16], and the Phenol-Explorer [17,18] have led to epidemiological studies precisely aiming to clarify the association between flavonoid intake and the prevalence and incidence of chronic diseases and cancers. According to the Phenol-Explorer database, the average intake of total flavonoids in France is 506 mg/day (with 51 mg/day of flavonols and 33 mg/day of flavones) [19], in the Mediterranean countries is 370.2 mg/day (with 24.8 mg/day of flavonols and 5.6 mg/day of flavones), and in the non-Mediterranean countries is 373.7 mg/day (with 29.5 mg/day of flavonols and 4.1 mg/day of flavones) [20]. This shows that the total daily consumption of flavonoids is higher in France than that in the other European countries. The same database indicates that 100 mL of red wine on average includes 88 mg of flavonoids, comprising anthocyanins (28 mg), dihydroflavonols (5.4 mg), flavanols (47 mg), flavanones (0.9 mg), and flavonols (6.9 mg) (Table 1), which may vary depending on the source and ageing, while white wine includes considerably less flavonoids (3.5 mg/100 mL). The USDA database for the flavonoid content of selected foods, release 3.3 (March 2018), reports that red wine includes 34.5–171.9 mg of flavonoids per 100 g, depending on the source [15].

Several epidemiological studies have reported a positive association between red wine intake and health. Individuals who consume moderate amounts of wine experience 20–30% reductions in all-cause mortality, particularly cardiovascular mortality [21], an effect known to be associated with the flavonoid composition of red wine [22]. The "French paradox" refers to the reduced cardiovascular mortality, due to higher intakes of red wine in France, when compared with other countries that consume similar amounts of saturated fats [23]. This preventive effect is considered to be based upon the strong antioxidant capacity of red wine flavonoids [11], since they react with the reactive compound of the radicals, and stabilize the reactive oxygen species [24,25].

Table 1. Contents of flavonoid family and major flavonoids in red wine.

Data Source	Phenol-Explorer (mg/100 mL) Mean (min~max) [18]	USDA (mg/100 g) Mean (min~max) [15]			
	Red Wine	Red Wine	Red Wine, Cabernet Franc	Red Wine, Cabernet Sauvignon	Red Wine, Syrah or Shiraz
Anthocyanins	27.78 (23.20~76.51)	19.27 (0.06~74.47)	55.09 (55.09)	35.59 (12.08~51.12)	152.98 (152.98)
Malvidin	15.62 (1.24~54.14)	13.84 (0.00~53.57)	44.09 (44.09)	26.24 (8.67~37.97)	121.65 (121.65)
Peonidin	1.81 (0.25~8.09)	1.25 (0.02~5.03)	2.40 (2.40)	1.85 (0.70~2.66)	7.82 (7.82)
Petunidin	2.36 (0.34~6.18)	1.98 (0.02~5.66)	4.70 (4.70)	3.32 (1.21~4.78)	14.16 (14.16)
Dihydroflavonols	5.44 (4.58~5.98)				
Dihydromyricetin	4.47 (4.47)				
Flavanols	47.02 (11.35~113.11)	11.08 (0~56.31)	15.41 (15.41)	18.36 (18.18~19.48)	16.79 (16.79)
(+)-Catechin	6.81 (1.38~39.00)	7.14 (0.00~39.00)	6.21 (6.21)	7.70 (6.90~8.18)	6.82 (6.82)
(−)-Epicatechin	3.78 (0.00~16.50)	3.79 (0.00~16.50)	9.20 (9.20)	10.66 (10.28~11.30)	9.97 (9.97)
Procyanidin	35.41 (9.86~55.87)				
Flavanones	0.85 (0.78~0.94)	2.40 (1.30~3.50)			
Naringenin	0.05 (0.04~0.07)	1.77 (1.03~2.51)			
Flavonols	6.86 (2.02~15.40)	1.57 (0~6.68)	0.77 (0.20~1.07)	0.89 (0.05~1.74)	2.11 (2.11)
Quercetin	3.10 (0.79~7.31)	1.04 (0.00~3.36)	0.62 (0.14~0.84)	0.58 (0.02~1.21)	2.11 (2.11)
Flavones		0.17 (0~0.56)	0.06 (0.01~0.13)	0.04 (0.01~0.11)	
Total	87.95	34.53	71.33	54.88	171.88

3. Anti-Inflammatory and Anti-Allergic Activities of Flavonoids Observed by In Vitro Experiments

The research provides evidence that oxidative stress is crucial in the airway and skin inflammation observed in asthma and atopic dermatitis patients, respectively [26,27]. The strong antioxidant capacity of flavonoids suppresses this allergic inflammation. Additionally, flavonoids are known to exert various ameliorative effects on allergic diseases [28,29].

Allergy is an IgE-mediated disease, pathologically comprising the sensitization and the effector phases. Flavonoids possess anti-allergic properties affecting both phases. Fewtress and Gomperts first identified the inhibition by flavones of transport ATPase in histamine release from rat mast cells [30]. Subsequently, flavonoids have been shown to inhibit the release of chemical mediators, such as histamine, hexosaminidase, and cyteinyl leukotrienes, by rat mast cells or human basophils [31–33]. In addition to the release of chemical mediators, mast cells and basophils can produce several cytokines associated with the late-phase allergic reaction. Meanwhile, flavonoids such as luteolin, quercetin, and baicalein were found to inhibit the synthesis of granulocyte macrophage-colony stimulating factor, tumor necrosis factor-α, and interleukin (IL)-6 production by the cultured mast cells in response to the cross-linkage of a high-affinity IgE receptor (FcεRI) [34,35]. IL-4 plays a major role in the sensitization phase since it stimulates the differentiation of B cells into IgE-producing cells and promotes the differentiation of naïve T cells into Th2 cells. Then, we examined the inhibitory effects of 45 kinds of flavonols and their related compounds on IL-4 synthesis, by analyzing the purified human peripheral blood basophils in response to cross-linkage of FcεRI [36–38]. Luteolin, apigenin, and fisetin showed the strongest inhibitory activity, with the half-maximal inhibitory

concentration (IC_{50}) value of these flavonoids for IL-4 synthesis ranging from 2.7–5.8 μM. Quercetin and kaempferol, meanwhile, had a moderate inhibitory effect on the IL-4 synthesis, with an IC_{50} value of 15.7–18.8 μM. Moreover, kaempferol was demonstrated to suppress the activation of IL-4 receptor-mediated signal transducers and activators of transcription, (STAT)6, by targeting Janus kinase (JAK)3 in the hematopoietic cell line [39]. Furthermore, epigallocatechin gallate, epicatechin gallate, gallocatechin gallate, anthocyanindin, delphinidin, and tricetinidin possess a pyrogallol function that suppresses the expression of FcεRI on human mast cells [40].

The aryl hydrocarbon receptor (AhR) is a receptor that leads to the toxic and biological actions of several aromatic environmental pollutants, such as dioxin [41]. In vitro bioassay of the dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)) revealed that flavonoids including apigenin, luteolin, baicalein, quercetin, kaempferol, and myricetin had significant inhibitory effects on the AhR activation, with an EC_{70} value (equal to 70% of the maximal response to TCDD) of 1.9–5.1 μM [42]. It has been demonstrated that the activation of AhR interferes with the differentiation of naïve CD4+ T cells into effector T cell subsets [43–46].

Nuclear factor-kappa B (NF-κB) is an important transcriptional factor that contributes pathologically to the development of various inflammatory diseases, including asthma, by inducing inflammatory responses, cell adhesion, and the anti-apoptosis process [47]. Flavonoids are also shown to suppress the NF-κB activation [48].

Autophagy is a cellular pathway that maintains cell homeostasis by eliminating the damaged cellular components, and its dysregulation may be associated with the development of various diseases [49]. The role of autophagy is also demonstrated in severe asthma, and flavonoids could potentially constitute the important adjuvants of conventional therapies for treating autophagy-related diseases [50].

4. Effects of Flavonoids on Allergic Diseases

As mentioned above, based on several anti-allergic activities of flavonoids, it is anticipated that an appropriate intake of flavonoids might prove beneficial in treating allergic diseases [51]. Indeed, the administration of flavonoids has revealed preventive or therapeutic effects in several allergy models.

We examined the preventive effect of astragalin (kaempferol 3′glucoside) on the onset or development of dermatitis by using NC/Nga mice, a model of atopic dermatitis [52]. The mice, which were administered a control diet, exhibited symptoms of dermatitis, scratching behavior, and serum IgE elevation along with aging, whereas the oral administration of astragalin (1.5 mg/kg) markedly prevented these symptoms [53]. Moreover, administrating an extract from the petals of *Impatiens balsamina* L., containing kaempferol 3-rutinoside and 2-hydroxy-1,4-naphthoquinone [54], prevented the development of dermatitis, while apigenin [55] and baicalein [56] therapeutically improved the severity of dermatitis in NC/Nga mice.

It was further demonstrated that in an ovalbumin (OVA)-sensitized asthmatic mouse model, the oral intake of luteolin (0.1 mg/kg) inhibited the bronchial hyper-reactivity and bronchoconstriction [57]. Moreover, it was reported that a polymethoxyflavonoid nobiletin, when administered at a dose of 1.5 or 5 mg/kg intraperitoneally to the OVA-sensitized rats, could reduce the number of eosinophils and the expression of eotaxin [58]. Subsequent investigations reported that numerous flavonoids such as quercetin, isoquercitrin, rutin, 3-*O*-methylquercetin 5,7,3′,4′-*O*-tetraacetate, narirutin, apigenin, luteolin, sulfuretin, hesperdin, fisetin, kaempferol, acacetin, silibinin, naringin, limonene, chrysin, genistein, skullcapflavone II, and anthocyanins indicated improvement in the asthmatic models [59]. Moreover, quercetin effectively quelled the anaphylactic reaction in the peanut-sensitized rats [60].

Several epidemiological studies have assessed the association of flavonoid intake with allergic diseases. A cohort study of the association between flavonoid intake and chronic diseases on 10,054 adults in Finland reported that the asthma incidence was lower with higher quercetin and hesperetin intakes [61]. A population-based case–control study performed in South London, UK, wherein 607 cases and 864 controls were enrolled, indicated that apple consumption was negatively

associated with asthma, whereas red wine intake was negatively associated with asthma severity [62]. The authors speculated that the associations between apple and red wine consumption and asthma might indicate a protective effect of flavonoids. However, there is a need to be careful as alcoholic drinks, particularly wines, have been shown to be associated with the triggering of asthma in respondents [63]. A subsequent study by the same research group, however, did not find any significant association of the dietary intake of catechins, flavonols, and flavones with the asthma prevalence and severity in a case–control study of 1471 adults in London [64]. The GA^{2}LEN (Global Allergy and Asthma European Network) study investigated the role of six major subclasses of flavonoids on ventilator function, with 2599 adults (aged 15 to 75 years) from nine European countries were enrolled [65]. The general consumption of 250 food types was estimated by the GA^{2}LEN food frequency questionnaire, and the intake of six major flavonoid subclasses; flavanones (eriodictyol, hesperetin, and naringenein), anthocyanins (cyaniding, delphinidin, malvidin, pelargonidin, petunidin, and peonidin), flavanols (catechins and epicatechins), flavonols (quercetin, kaempferol, myricetin, and isohamnetin), flavones (luteolin and apigenin) and polymers (proanthocyanidins, theaflavins, and thearubigins), and proanthocyanidins was calculated using the USDA database. The average of the total flavonoid intake was 291.2 mg/day and it varied among people from the nine countries (from 231.7 mg/day in Germany to 817.3 mg/day in Poland), whereas the intake of proanthocyanidins was 154.6 mg/day. Among the total food and beverage consumption, wine and beer together contribute to about 21% and 14.9% of the total flavonoid and proanthocyanidin intake, respectively. A lower prevalence of forced vital capacity (FVC) below the lower limit of normal and a higher ratio between forced exhaled volume in 1 second (FEV_1) and FVC (FEV_1/FVC) was observed in those with higher total flavonoid and proanthocyanidin intakes.

Nevertheless, flavonoid intervention in humans is limited. Previous clinical research using several flavonoid extracts indicates that flavonoids have therapeutic effects on allergic rhinitis [66–70]. These extracts were *Perilla frutescens* (rosmarinic acid as a major flavonoid), apple polyphenols (procyanidins or apple condensed tannin, catechin, epicatechin, phlorizin, and chlorogenic acid), hop water extract (quercetin and kaempferol glycosides), and tomato extract (naringenin chalcone). A summary of these flavonoid intervention studies in allergic rhinitis is shown in Table 2. Enzymatically-modified isoquercitrin (EMIQ) is a quercetin glycoside comprising isoquercitrin and its maltooligosaccharides, which markedly enhances the bioavailability. We performed clinical research to examine the efficacy of EMIQ on patients with Japanese cedar pollinosis in 2007 and 2008 [71,72]. In a double-blind, placebo-controlled design, the patients were randomly assigned to the EMIQ group or the placebo group. The 2007 study commenced after the pollen dispersion, and thus we examined the therapeutic effect of EMIQ, whereas the 2008 study commenced 3 weeks before the first day of pollen dispersion, to evaluate the preventive effect of EMIQ on the symptoms of pollinosis. The daily intake for these studies was 100 mg EMIQ for 8 weeks. The total symptom (nasal and ocular symptoms) scores for the EMIQ groups in the 2007 and 2008 trials were optimally lowered by 48% and 33%, respectively, compared with the scores for the placebo groups, indicating a substantial ameliorative effect of EMIQ. A randomized clinical trial of silymarin demonstrated its ameliorative effect on the symptoms of allergic rhinitis [73]. Moreover, a randomized, double-blind, placebo-controlled study of pycnogenol, a proprietary mixture of water-soluble bioflavonoids extracted from the French maritime pine, which contains proanthocyanidines, revealed its ameliorative effect on seasonal allergic rhinitis [74].

Pycnogenol was also demonstrated to be effective in treating asthma. The first study was performed to evaluate the effect of pycnogenol on asthma in a randomized, double-blinded, placebo-controlled, crossover design, in which 26 asthmatic patients were enrolled [75]. These patients were randomly assigned to receive either 1 mg/lb/day (maximum 200 mg/day) pycnogenol or a placebo for 4 weeks and were then crossed over to the other regimen for the next 4 weeks. Twenty-two patients who completed the study responded positively to pycnogenol. Subsequently, in a randomized, placebo-controlled, double-blind study involving 60 asthmatic patients, aged 6–18 years, compared with the placebo group, the pycnogenol group revealed significantly greater improvement in the lung function and asthmatic

symptoms, which resulted in the reduced or discontinued use of rescue inhalers [76]. Another study, which evaluated the effect of pycnogenol on the allergic asthma management of patients for 6 months, also revealed a favorable result [77]. In this study, pycnogenol at 100 mg/day proved to be effective in controlling the symptoms of allergic asthma and reduced the need for medication.

Table 2. Clinical studies of flavonoids in allergic rhinitis.

Test Product	Major Flavonoid(S)	Study Design	Primary Endpoint	Ref.
Extract of *Perilla frutescents*	Rosmarinic acid (50 mg/day or 200 mg/day)	A 21-day randomized, double-blind, placebo-controlled study (n = 29)	A significant increase in responder rates for total symptoms related to seasonal allergic rhinoconjunctivitis	[66]
Apple polyphenols (500 mg/day)	Procyanidins, tannin, catechin, epicatechin, phlorizin, and chlorogenic acid	A 12-week randomized, double-blind, placebo-controlled study (n = 36)	A significant reduction in the sneezing score related to Japanese cedar pollinosis	[67]
Apple polyphenols (50 mg/day or 200 mg/day)	Procyanidins, phenol carboxylic acids	A 4-week randomized, double-blind, placebo-controlled study (n =33)	Significant improvements in sneezing attacks and nasal discharge in the 200 mg group and in sneezing attacks in the 50 mg group, related to persistent allergic rhinitis	[68]
Hop water extract (100 mg/day)	Quercetin, kaempferol glycosides	A 12-week randomized, double-blind, placebo-controlled study (n =39)	A significant difference in the symptom score and the symptom plus medication score related to Japanese cedar pollinosis 10 weeks after the intervention	[69]
Tomato extract (360 mg/day)	Naringenin chalcone	An 8-week randomized, double-blind, placebo-controlled study (n =33)	A significant decrease in the total symptom score related to perennial allergic rhinitis	[70]
EMIQ (100 mg/day)	Quercetin glycoside	An 8-week randomized, double-blind, placebo-controlled study (n = 20) (therapeutic design)	A significant decrease in the ocular symptom score related to Japanese cedar pollinosis	[71]
EMIQ (100 mg/day)	Quercetin glycoside	An 8-week randomized, double-blind, placebo-controlled study (n = 24) (preventive design)	A significant decrease in the ocular symptom plus medication score related to Japanese cedar pollinosis	[72]
Silymarin (420 mg/day)	Silibinin, silydianine, and silychristine	A 1-month randomized, double-blind, placebo-controlled study (n = 60)	A significant improvement in the clinical symptom severity related to allergic rhinitis	[73]
Pycnogenol (100 mg/day)	Proanthocyanidine	A 5 to 8-week randomized, double-blind, placebo-controlled study (n = 39) (preventive design)	Lower scores for the eye (−35%) and nasal (−20.5%) symptoms related to birch pollinosis	[74]

EMIQ, enzymatically modified isoquercitrin.

5. Future Perspectives of Red Wine Flavonoids for Allergic Diseases

A direct interventional study evaluating the beneficial effects of red wine flavonoids on allergic diseases has not been performed to date. However, as described elsewhere, one epidemiological study reported that red wine intake was negatively associated with asthma severity and suggested that flavonoids may produce a protective effect on asthma. Red wine is a major source contributing to the daily flavonoid intake for wine lovers, thus possibly ameliorating the allergic symptoms. However,

careful attention is required in clinical trials, as wine is a triggering factor for worsening symptoms in certain asthmatic patients and heavy wine consumption is accompanied by alcohol intake that is not good for health and behavior [78].

Table 3 summarizes the anti-allergic effects of flavonoids. Flavonoids possess antioxidant, anti-inflammatory, anti-allergic, and immunomodulating effects. Several studies have reported the benefits of flavonoids in allergic models, however, the evidence in the epidemiological studies and clinical studies is presently limited. Future studies are needed, to focus on whether an appropriate intake of flavonoids can constitute a dietary contribution in the prevention and amelioration of allergic diseases.

Table 3. Summary of the anti-allergic effects of flavonoids.

1. Biological properties
Antioxidant [9,10,13,24,25], anti-inflammatory [9,10,13,24,31,48], anti-allergic [28–40], and immune-modulating activities [31,40,42]
2. In vivo effects in animal models
Preventative and therapeutic beneficial effects of various flavonoids in several allergic models [53–60]
3. Epidemiological study
An increase of flavonoid intake is suggested to be beneficial for respiratory function [61,62,64,65]
4. Intervention study
Some kinds of flavonoids are efficacious for allergic rhinitis [66–74]
Pycnogenol is efficacious for asthma [75–77]

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Review

Strategic Syntheses of Vine and Wine Resveratrol Derivatives to Explore Their Effects on Cell Functions and Dysfunctions

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Abstract: *Trans*-resveratrol, the most well-known polyphenolic stilbenoid, is found in grapes and accordingly in wine and it is considered to be beneficial for human health, especially towards the aging-linked cell alterations by providing numerous biological activities, such as anti-oxidant, antitumoral, antiviral, anti-inflammatory, neuroprotective, and platelet anti-aggregation properties. Although *trans*-resveratrol is a promising molecule, it cannot be considered as a drug, due to its weak bio-availability and fast metabolism. To overcome these weaknesses, several research teams have undertaken the synthesis of innovative *trans*-resveratrol derivatives, with the aim to increase its solubility in water and pharmacological activities towards cell targets. The aim of this review is to show the chronological evolution over the last 25 years of different strategies to develop more efficient *trans*-resveratrol derivatives towards organism physiology and, therefore, to enhance various pharmacological activities. While the literature on the development of new synthetic derivatives is impressive, this review will focus on selected strategies regarding the substitution of *trans*-resveratrol phenyl rings, first with hydroxy, methoxy, and halogen groups, and next with functionalized substituents. The effects on cell functions and dysfunctions of interesting resveratrol analogs will be addressed in this review.

Keywords: resveratrol derivatives; synthesis strategies; substituents phenyl rings; biological targets; efficacy towards diseases

1. Introduction

Polyphenolic compounds produced by vine belong essentially to flavonoids, stilbenoids, and anthocyanins, and are distributed in leaves, berries (seeds and skin), and lignified tissues. In the plant, they either play the role of phytoalexins (flavonoids and stilbenoids) [1,2] or are responsible for the color in leaves, flowers, and berries (anthocyanins) [3]. In addition, in each series, at least one polyphenolic compound provides health-promoting effects on humans. [4–6]. We were interested in *trans*-resveratrol (**1**, Figure 1), the leader in the polyphenolic stilbenoid series, present not only in vine, grapes, and, accordingly, in wine [7], but also in numerous other plants, including the Asiatic plant, *Polygonum cuspidatum* [8]; edible plants, such as peanuts [9]; and red fruit [10]. Accordingly, *trans*-resveratrol is part of our daily diet and this is a precious chance for our health because this molecule provides numerous biological activities, such as anti-oxidant [11], antitumoral [12], antiviral [13], and anti-inflammatory activities [14]. In addition, *trans*-resveratrol extents longevity [15], induces cell pro-differentiation [16,17], is a neuroprotective agent [18], and acts against platelet aggregation [19]. Cell targets have already been identified, such as membrane receptors, tyrosine

kinases, phosphatases, sirtuins, and p53 anti-oncogene [20]. In addition, the interaction of resveratrol with tyrosyl transfer-RNA (tRNA) synthetase (TyrRS) may induce poly(ADP-ribose) polymerase 1 (PARP1) activation in cell nuclei in mice [21]. These various biological activities are often related to the anti-oxidant nature of resveratrol, itself explained in part by the ease of transfer of hydrogen atoms from the three phenolic groups to cellular species to act on adverse effects [22].

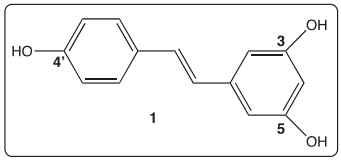

Figure 1. Structure of *trans*-resveratrol (**1**).

Since its discovery in 1940 [23], *trans*-resveratrol has been the subject of more than 20,000 publications that describe the different methods to obtain it (extraction from plants [24], synthetic ways [25], enzymatic syntheses [26]) and its numerous biological activities [27]. So, the regular consumption of food and moderated wine containing this health-beneficial molecule may be an effective way to prevent some diseases. In contrast, *trans*-resveratrol cannot be considered directly usable as a drug because of its weak bio-availability due to its low water solubility [28]. To overcome these difficulties, several research teams have undertaken the synthesis of new *trans*-resveratrol derivatives in the aim to enhance bio-availability and pharmacological activities. Previously, several reviews have stated a part of these studies by insisting either on synthetic schemes and biochemical activities [29] or on the pharmacological activities of new stilbene derivatives only [30–33]. Hence, this review will specifically focus on the chronological evolution for the last 25 years of different strategies followed by researchers to develop very efficient *trans*-resveratrol derivatives exhibiting various pharmacological activities. As in the case of *trans*-resveratrol, the numerous publications regarding synthetic *trans*-resveratrol derivatives are impressive. Indeed, a large panel of structural modifications could be achieved on the parent molecule, such as addressing the nature, the number, and the position of the phenyl rings' substituents, the nature of the aryl ring, i.e., phenyl vs replacement of a phenyl ring by another aromatic one, the replacement of the C=C double bond by a diazo or imine bond, or an isosteric heterocyclic ring. It turns out that it is difficult to list all the derivatives and their diverse biochemical activities in a single review. Hence, this review will specifically focus on pharmacological improvements resulting from structural modifications performed at the phenyl ring substituents.

2. Which Strategies to Modify *trans*-Resveratrol

The molecular structure of *trans*-resveratrol (**1**, Figure 1) is a stilbene core made of two phenyl rings linked by a double bond. Three hydroxy groups are present in both phenyl rings in position 3, 4′, and 5 (Figure 1). Their pKa values in aqueous medium are 9.8, 8.8, and 11.4, respectively [34]. The sensitive point of the molecule is the double bond separating the two phenyl rings that can be easily isomerized under light, knowing that isomer *E* of resveratrol is the biological active form [35]. Apart from this, *trans*-resveratrol is a non-toxic and air stable molecule, in the form of a white powder it has a melting at 261 °C; is soluble in ethanol, acetone, and tetrahydrofuran; and poorly soluble in water [36]. So, the chemical transformations of *trans*-resveratrol can be easily considered; they essentially take place at the phenolic functions that are transformed into ether or ester functions [37]. However, the access to new derivatives from *trans*-resveratrol itself sets limits to create innovative bio-active polyphenolic analogs. Fortunately, the essential stilbene core of resveratrol is easily accessible by different chemical methods, including Perkin [38], Wittig [39,40], Horner-Wittig-Emmons [41], Heck [39], and Suzuki [42] reactions (Figure 2). Each approach starts from different starting materials, which are usually commercially available and most of them are cheap.

So, the library of *trans*-resveratrol derivatives that has been synthesized over the last 25 years is quite impressive. Over time, some new derivative structures have become more complex in order to move towards more selective and effective biological activities.

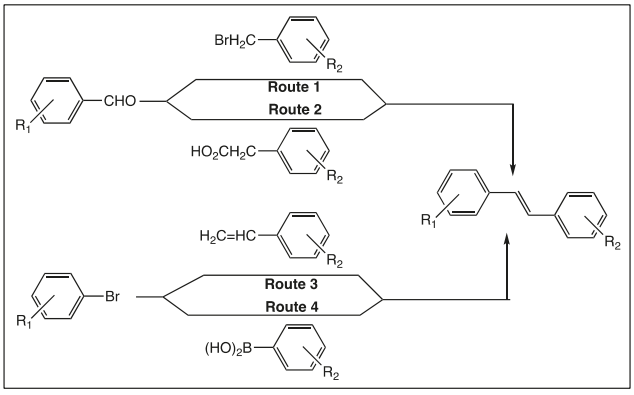

Figure 2. Principal synthetic methods for obtaining stilbene derivatives. Route 1: Wittig method [39,40], route 2: Perkin method [38], route 3: Heck method [39], route 4: Suzuki method [42].

3. Phenyl Rings Substitution of *trans*-Resveratrol by Hydroxy, Methoxy, and Halogen Groups

The biological activities of natural *trans*-resveratrol derivatives in vines, such as pterostilbene (**2**), piceatannol (**3**), and resveratrol oligomeric analogs as *trans*-ε-viniferin (**4**, Figure 3), are comparable to that of resveratrol (**1**) [43–47]. Thus, several research groups have used such bio-active molecules as an inspiration to synthesize numerous hydroxylated or/and methoxylated stilbenes [48–50].

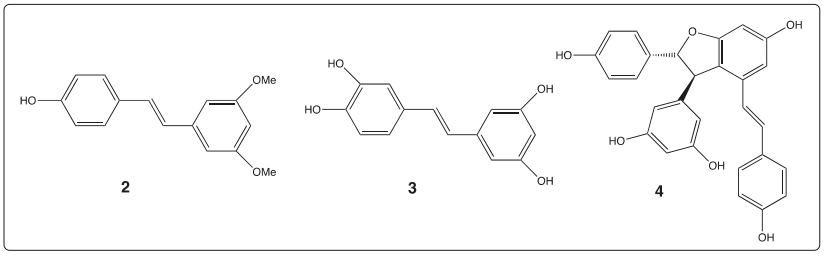

Figure 3. Structure of natural *trans*-resveratrol derivatives: Pterostilbene (**2**), piceatannol (**3**), and *trans*-ε-viniferin (**4**).

Since the early 2000s, most research works have focused more specifically on non-natural resveratrol derivatives bearing hydroxy and/or methoxy groups and/or halogen atoms as substituents. Lately, a review summarized the manifold therapeutic activities of some of these polyphenolic derivatives [32]. In the conclusion, the authors of this review pointed out the fact that a structure-activity relationship study was missing. Indeed, it is difficult to predict pharmacological activities of this series of derivatives because changing one substituent may affect the biochemical property. In addition, as in the case of *trans*-resveratrol, one derivative may provide several biochemical properties. Thus, in this part, we will focus our discussion on a few examples of this type of resveratrol derivatives to illustrate the fact that it is often necessary to synthesize a large number of hydroxylated, methoxylated, and/or halogenated stilbenes to find good candidates for a particular therapy disease.

Increasing the number of hydroxy groups on the resveratrol phenyl rings is already a good starting point to enhance pharmacological activities [48]. Thus, the two pyrogallol groups in

3,4,5,3′,4′,5′-hexahydroxystilbene (**5**, Figure 4) synthesized by Murias's group appear to provide various activities for this resveratrol derivative, such as COX-2 inhibition correlated with a docking approach [51]; anti-oxidant activity through ortho semi-quinones formation [52], which triggers cytotoxic activity against breast cancer cells mediated by induction of p53 and downregulation of mitochondrial superoxide dismutase [53]; and oxidative stress in cancer cells [54]. Furthermore, resveratrol derivative **5** is a potent Human Immunodeficiency Virus (HIV-1) inhibitor at micromolar range [55].

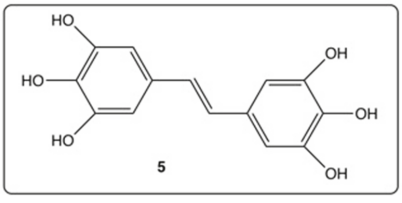

Figure 4. Structure of 3,4,5,3′,4′,5′-hexahydroxystilbene (**5**) bearing two pyrogallol groups.

In contrast, 3,4,5,4′-tetramethoxystilbene or DMU-212 (**6**, Figure 5) is only substituted by methoxy groups and may provide antitumoral activities, as described by different research groups. By selectively targeting the mitochondria of transformed lung fibroblasts, W138VA, DMU-212 (**6**) inhibited the cell growth (IC_{50} = 0.5 μM) compared with resveratrol (IC_{50} = 50 μM) [56]. Apoptotic induction and metastatic inhibition in melanoma cells by DMU-212 was highlighted too [57]. In in vivo experiments, injection of DMU-212 in male Wistar rats (rat hepatocarcinogenesis) allowed Murias's group to prove that compound **6** may modulate the activation of NF-κB, AP-1, and STAT3 transcription factors [58]. Given the absence of hydroxy groups, an antioxidative activity cannot be invoked and the cell signaling pathway should be highlighted. By this way, it was found that another derivative bearing only methoxy groups, the *trans*-3,4′,5-trimethoxyresveratrol (**7a**, Figure 5), inhibited cancer cell growth (HeLa cells) by inhibiting tubulin polymerization [59]. In addition, the cis-3,4′,5-trimethoxyresveratrol (**7b**, Figure 5) was a very potent cell proliferation inhibitor and acted at the tubulin cholchicin binding site [60]. From these three last derivatives, **6**, **7a**, and **7b**, the presence of an additional methoxy group can modify the inhibition potencies, while the configuration of the double bond did not change it.

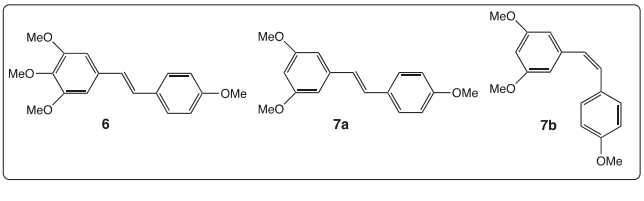

Figure 5. Structure of DMU-212 (**6**), *trans*-3,4′,5-trimethoxyresveratrol (**7a**), and cis-3,4′,5-trimethoxyresveratrol (**7b**).

These two opposite examples of *trans*-resveratrol derivatives **5** and **6** show that hydroxy and methoxy groups may afford specific chemical properties, such as an improvement of the lipohilicity and bio-availability, promotion of interactions with amino acids in the receptor pocket [61,62], and induction of semi-quinones formation [52], which may induce specific pharmacological properties. Therefore, the combination of these two oxygenated groups, to which halogen atoms are possibly added, widens even more the field of pharmacological properties of these stilbenes. For example, Csuk's team reported the biological activities of more than 100 stilbenes substituted with hydroxy and/or methoxy groups and/or fluorine atom only [63–67]. Throughout Csuk's five publications, it appears that compounds **8**–**12** (Figure 6) provided antitumoral activity [64], acetylcholinesterase

and butyrylcholinesterase inhibitions [65], anti-oxidant activity [66], and oxidant stress decrease in Caenorhabditis elegans [67].

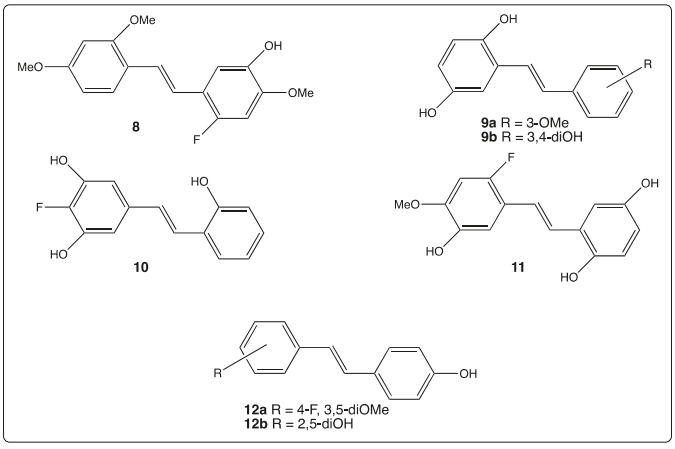

Figure 6. Structure of compounds **8**–**12**.

Generally, synthetic *trans*-resveratrol derivatives are tested for their potential therapeutic properties and rarely for their antimicrobial activities. However, among the library of stilbenoids of Csuk's team, 25 compounds were evaluated for their antibacterial and antifungal activities [63]. They were divided in three series **13a**, **13b**, and **13c** in which the R substituent is a fluorine atom, or/and a hydroxy or a methoxy group (Figure 7). It turned out that position 4 with respect to the hydroxy group in compounds **13a** was more favorable than position 2 or 3 of the same group in compounds **13b** and **13c**.

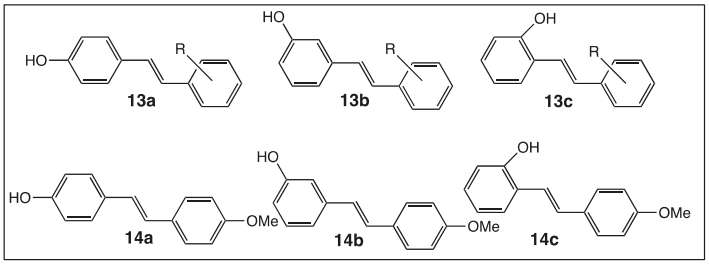

Figure 7. Structure of 4-hydroxy, 3-hydroxy, and 2-hydroxystilbenes **13a–c** and **14a–c**.

In another study, 4-hydroxy-4′-methoxystilbene (**14a**, Figure 7) provided no antimicrobial activity towards two grapevine pathogens (Botrytis cinerea and Plasmopara viticola), while compounds **14b** and **14c** (Figure 7), both isomers of **14a**, showed an activity superior to those of *trans*-resveratrol and pterostilbene [68]. In contrast, in the case of antitumoral tests, the results are reversed: On the one hand, stilbene **14a** appeared to be a better candidate than *trans*-resveratrol for the inhibition of human colorectal tumor cells SW480, and on the other hand, isomer **14b** showed a weaker activity than the parent molecule [69].

4. Phenyl Rings Substitution of *trans*-Resveratrol by Functionalized Groups or Bioactive Moieties

The selected examples mentioned in the previous part show too many possibilities to dream up new *trans*-resveratrol derivatives as well as difficulties to predict their pharmacological activities. Since the late 2000s, several studies have shown that, while keeping the basic structure of *trans*-resveratrol, it remains possible to develop interesting derivatives starting from resveratrol (bioactivities of which are well defined) and adding judicious moieties, enabling enhancement of the bio-availability or to increase a particular biochemical property. As a result, therapeutic activities of these *trans*-resveratrol derivatives are better targeted.

Few examples of *trans*-resveratrol derivatives directly substituted on one of the aromatic carbon atoms have been described. Indeed, these substitution reactions cannot be carried out directly on *trans*-resveratrol itself and their syntheses require several chemical steps. However, a hybrid compound **15** named resveratrol fatty alcohol or RFAs (Figure 8) reported in 2007 results from the combination of a fatty alcohol and *trans*-resveratrol, which have neuroregenerative activity and neuroprotective features, respectively [70]. Cumulative effects at both parts in conjugate **15** provided a higher bio-activity than its parent moieties, polyphenol and fatty alcohol. In an inventive study [71], the *trans*-resveratrol structure was preserved and both ortho positions of 4-hydroxy group (responsible of anti-oxidant activity) were substituted with bulky electron donating groups in **16a** and **16b** (Figure 8). Adding two bulky substituents to the *trans*-resveratrol structure allowed enhancement of the anti-oxidant activity while strongly reducing interferences with estrogen and ArH receptors [71].

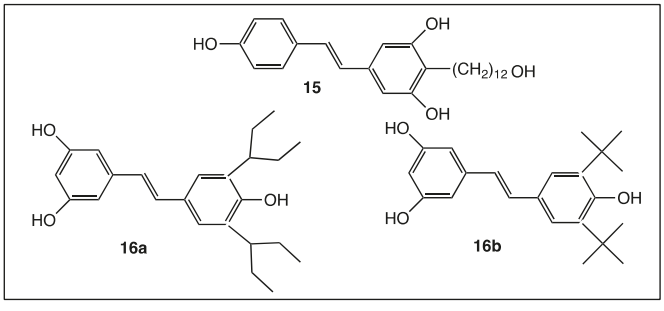

Figure 8. Structure of Resveratrol Fatty Alcohol (RFAs) (**15**) and compounds **16a** and **16b**.

The presence of chemical functions, such as ethers, carboxylic acids, esters, and amides, on the *trans*-resveratrol core may modify its lipophilic character and induce mechanisms in the cellular environment, which leads to the provision of better biological activities. The addition of various functions or alkyl chains could be carried out directly by *O*-acylation or *O*-alkylation reactions of commercially available *trans*-resveratrol. Therefore, the simple resveratrol aliphatic acid **17** (Figure 9) is more soluble in water than the parent molecule and inhibits the expression of TLR-2 [72]. Pterostilbene aliphatic amine **18** (Figure 9) was considered as a multitarget-directed agent for the therapy of the Alzheimer's disease because it induced inhibition, although at a micromolar range of Aβ aggregation, and displayed moderate cholinesterase inhibition activity and acceptable inhibitory activity towards MonoAmine Oxidase (MAO) [73].

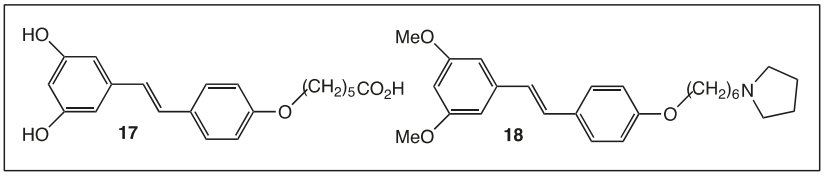

Figure 9. Structure of resveratrol aliphatic acid **17** and resveratrol aliphatic amine **18**.

The bio-availability of *trans*-resveratrol was enhanced upon its transformation into tri-esters **19a** and tri-ethers **19b** (Figure 10) [74]. Improvement of this feature in these compounds led to a therapeutic interest (melanogenesis inhibition) and cosmetics application. Mono and diesters resveratrol derivatives **20a** and **20b** (Figure 10) were recently evaluated for their anti-oxidant activity and their possible use in food and biochemical systems [75]. While referencing to Biasutto's work [76], the authors suggested that upon crossing the cell membrane barrier, the esters were hydrolyzed thus releasing resveratrol, which acts as an antioxidant agent.

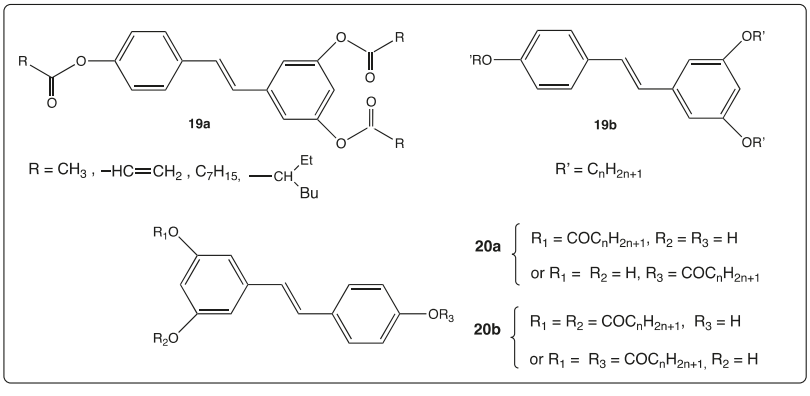

Figure 10. Structure of mono, di, tri ethers and esters **19a–b** and **20a–b**.

In the past ten years, the multi-targeted designed drugs (MTD's) paradigm (that emerged especially in the fields of neurodegenerative diseases and cancers [77]) has consisted in designing hybrid compounds from at least two molecules providing complementary therapeutic activities. Hybridization of a rich bio-active molecule, such as *trans*-resveratrol, with a known pharmacophore has allowed researchers to better target biological activities. Given the good reactivity of phenolic functions, this concept has been easily applied to synthesize hybrid compounds. *O*-alkylation of one or two phenolic functions with a PPARα agonist, such as fenofibric acid (**21,** Figure 11), led to compounds **22a** and **22b** (Figure 11), lowering triglycerides in hyperlipidemic mice and blood glucose levels in KKAy mice, respectively [78].

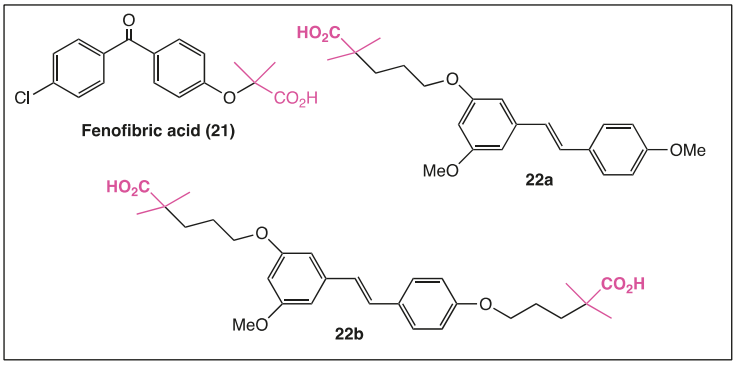

Figure 11. Structure of fenofibric acid (**21**) and hybrid compounds **22a**–**b**.

1,3,4-Oxadiazole is a heterocyclic moiety with potential antitumoral activity if this one is part of a molecular bioactive structure. Therefore, hybridization of 1,3,4-oxadiazole and *trans*-resveratrol by an amide or an ester bond allowed Murty's group to develop an inventive series of drug-like molecules, including **23a** and **23b** (Figure 12), that provided a dual therapeutic effect towards human cancer cell lines, SiHa, MDA-MB-231, and PANC-1, which turned out to be higher than that of polyphenol [79].

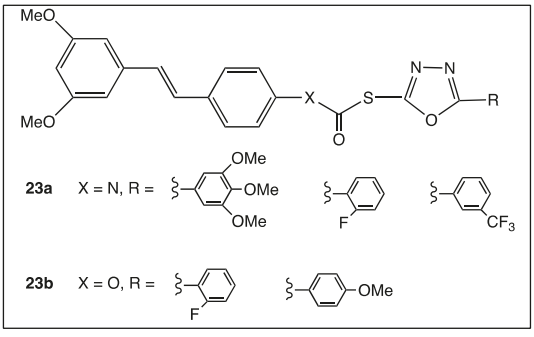

Figure 12. Structure of resveratrol-oxadiazole hybrid compounds **23a**–**b**.

The presence of a carboxylic group in nonsteroidal anti-inflammatory drugs, such as ibuprofen (**24,** Figure 13), is responsible for gastrointestinal toxicities. In contrast, *trans*-resveratrol has a protective effect against gastric mucosa damage. Therefore, linking this polyphenol and ibuprofen together by esterification reaction led to a hybrid compound **25** (Figure 13), which may solve gastrointestinal problems while keeping the anti-inflammatory activity of the ibuprofen moiety [80].

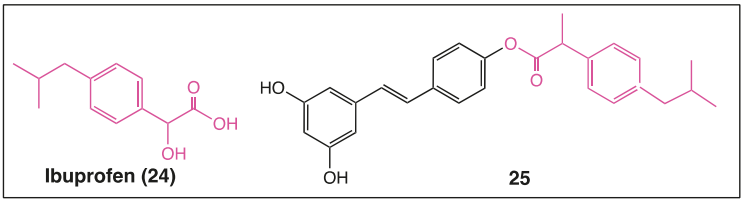

Figure 13. Structure of ibuprofen (**24**) and resveratrol-ibuprofen hybrid compound **25**.

To mitigate the weak bio-availability and the unfavourable pharmacokinetic properties of *trans*-resveratrol, various bio-compatible resveratrol-loaded particles have been successfully developed [81]. Another way is to synthesize resveratrol derivatives bearing a moiety capable of promoting the crossing of the membrane barrier. In 2012, Sciuto's team studied the interactions of two hydrophobic *O*-phosphorylresveratrol derivatives **26a** and **26b** (Figure 14) with a DMPC (1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine) model membrane [82]. 3-*O*-phosphorylresveratrol derivative (**25a**) turned out to insert into the hydrophobic core of the membrane and diffused across it, while isomer **26b** was preferentially bound to the membrane surface and did not cross the membrane barrier. These results were correlated with the fact that the antitumoral effect of **26a** against DU-145 prostate cancer cells was higher than that of **26b**.

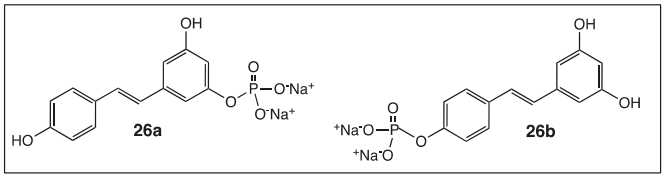

Figure 14. Structure of *O*-phosphorylresveratrol derivatives **26a**–**b**.

The same research team achieved the direct coupling of a lipophilic group (related to lipids membrane) to resveratrol derivatives **26a** and **26b** to afford amphiphilic resveratrol lipoconjugates **27a** and **27b** (Figure 15) [83]. These innovative *trans*-resveratrol derivatives had greater anticancer activity against the neuroblastoma SH-SY5Y cell line than the free parent molecule. Lately, a mixture of *O*-phosphorylresveratrol derivatives **26b** and amphiphilic resveratrol lipoconjugate **27b** was shown to be efficient to abolish hIAPP amyloid growth and membrane damage in diabetes mellitus type II pathology [84]. Both *trans*-resveratrol derivatives act in a complementary way to fight amyloid poration phenomena and lipid extraction by amyloid fibrils.

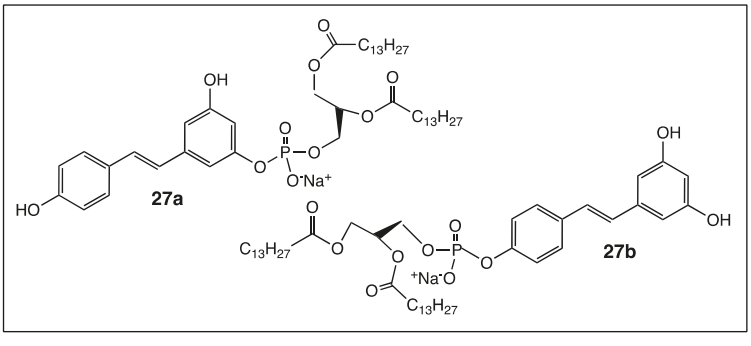

Figure 15. Structure of resveratrol-1,2-DiMyristoyl-*sn*-glycero-3-Phosphoholine (DMPC) hybrid compounds **27a**–**b**.

5. Discussion

The main goal of research teams in designing new resveratrol derivatives is the improvement of one or several biological activities of the parent molecule. These improvements involve not only the way to "dress" the stilbene scaffold, but also to address both the pharmacokinetics and bioavailability aspects. In this review, we only considered stilbene derivatives whose modifications relate to the nature, the number, and the position of aromatic rings' substituents. We showed the chronological evolution over the last 25 years of different chemical strategies followed by researchers in the aim to

develop efficient *trans*-resveratrol derivatives towards various pharmacological activities. It should be noted especially the evolution of derivatives bearing non-functionalized phenyl rings' substituents to derivatives designed according to the multi-targeted designed drugs (MTD's) paradigm. Because the number of publications related to such *trans*-resveratrol derivatives is impressive, the list of these relevant cited papers is far from exhaustive. However, among the selected examples in this review, the following conclusions may be raised based on the points summarized below.

First, even the number and position of the 9 hydroxy groups on resveratrol phenyl rings play an important role in the various activities of the polyphenols, and the presence of methoxy groups and/or halogen atoms may lead to interesting properties. Thus, the increase of the number of hydroxy groups on the resveratrol phenyl rings (Figure 4) enhances COX-2 inhibition, anti-oxidant activity, and cytotoxic effect against breast cancer [51–54]. Stilbenes **8**–**12** (Figure 6) bear both the hydroxy and methoxy groups and fluorine atoms that provide antitumoral activity [64], acetylcholinesterase and butyrylcholinesterase inhibition activities [60], and anti-oxidant activity [66]. In the other hand, in compound **14a**, the position 4 of the hydroxy group is less favorable than the positions 2 or 3 in compounds **14b** and **14c** (Figure 7) for antibacterial and antifungal activities [68] while the presence of the methoxy group and/or fluorine atom on the other phenyl ring of stilbene **13a** (Figure 7) reverses this result [69]. When 4-hydroxy group is surrounded by two bulky groups in stilbenes **16a** and **16b** (Figure 8), the anti-oxidant activity is enhanced, while strongly reducing its interferences with estrogen and ArH receptors [71]. However, the tetra-methoxylated stilbene DMU-212 (**6**, Figure 5) leads to an increase in antitumoral activity by apoptotic induction [56–58].

Thus, as a result of so many complex results, it appears that applying the multi-targeted designed drugs (MTD's) paradigm may be a very promising concept to better identify judicious stilbene derivatives with interesting pharmacological activities. Indeed, innovative coupling of *trans*-resveratrol with a fatty alcohol provided resveratrol fatty alcohol or RFAs (**15**, Figure 8) bearing both neuroregenerative and neuroprotective features [70]. This hybrid compound may be considered as the premise of a large series of stilbenes designed according to the multi-targeted designed drugs (MTD's) paradigm. Thus, the *O*-alkylation of one or two phenolic functions with a PPARα agonist, such as fenofibric acid (**21**), leads to hybrid compounds **22a** and **22b** (Figure 11), lowering triglycerides in hyperlipidemic mice and blood glucose levels in mice [78], respectively. Coupling ibuprofen (**24**) with resveratrol solves the side effect problem because the resveratrol moiety **25** (Figure 13) protects the gastric mucosa against the acidity of the anti-inflammatory drug [80].

In a last point, the lipophilic character of resveratrol is a crucial parameter to increase its biological activities. It can be modulated in one way or another depending on the nature of the added chemical functions (ethers, carboxylic acids, esters, amides, etc.) to the *trans*-resveratrol core. For example, a series of resveratrol aliphatic acids, including compound **17** (Figure 9), synthesized in 2008 proved to be more soluble in water than the parent molecule and, therefore, the binding affinity of **17** to human serum albumin was 40-fold higher [85]. It was recently shown that the mono-O-phosphorylresveratrol derivatives **26a** and **26b** (Figure 14) have a hydrophobic character. As a result, their interaction with DMPC model membrane turned out to be good [82]. In contrast, tri-esters **19a** and tri-ethers **19b** (Figure 10) have higher lipophilic characters than resveratrol and may be considered as good candidates for skin-whitening cosmetics [74].

Biochemical mechanisms and lipophilic aspects of resveratrol derivatives are overall well highlighted in the literature cited in this review. However, as it was mentioned in a recent review [33], we noticed that most of the biological tests carried out on resveratrol derivatives bearing hydroxy, methoxy, and halogen groups have been done on cultured cell lines (in vitro) or on isolated enzyme, but rarely in vivo and never through clinical studies. However, in vivo experiments with resveratrol derivatives bearing functionalized substituents have been carried out, but the pharmacokinetics aspects were not mentioned [78].

6. Conclusions

To get a better understanding of the biological effects of natural *trans*-resveratrol either from vine grape derived-beverages or from diet, the use of resveratrol derivatives appears very useful for the identification of cell targets to help maintain the best healthy conditions, e.g., to prevent diseases, such as stroke, cancer, and infection, and to increase longevity. Some resveratrol derivatives may allow differentiation of candidates with or without anti-oxidant properties. From a pharmaceutical point of view, the discovery of innovative resveratrol analogs is also very relevant to determine effective and safe dosage. Moreover, this requires more in vivo experiments to understand the metabolism of the derivatives and effects on whole organisms in terms of benefits and possible toxicity.

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

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diseases

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Review

Polyphenol Extracts from Red Wine and Grapevine: Potential Effects on Cancers

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Abstract: Wine has been popular worldwide for many centuries and currently remains an important component of our diet. Scientific interest in wine and its health effects has grown considerably since the 1990s with the emergence of the "French Paradox" concept, correlating moderate wine consumption, a characteristic of the Mediterranean diet, and low incidence of coronary heart diseases. Since then, the positive effects on health, health promotion, disease prevention, and disease prognosis of moderate wine consumption, in particular red wine, have been attributed to its polyphenolic compounds such as resveratrol, quercetin, and other flavonoids acting as antioxidants. Several epidemiological, in vivo and in vitro, studies have reported that moderate red wine or red wine polyphenolic extract consumption may be active in the prevention and treatment of chronic diseases such as cardiovascular disease, metabolic syndrome, degenerative pathologies, and cancer. The aim of this review is to summarize the current findings about the effects of red wine polyphenols on cancer and to discuss how the polyphenolic composition of red wine may influence its chemopreventive properties.

Keywords: red wine; polyphenols; cancers; colorectal

1. Introduction

Wine has been produced since the beginnings of civilization, presumably starting in the Near East; traces are found in the Egyptian hieroglyphs, the code of the Babylonian king Hammurabi, and in Assyrian bas-reliefs. The influence of wine on the development of most Eurasian societies is considerable and it is the only food for which such a sacred and symbolic character has been attributed. Since antiquity, wine has been an important part of the diet of most countries around the Mediterranean. It is consumed in several forms, and it is also a prime commercial activity. Today, wine consumption, even though it has been decreasing over the past several decades, remains important in the countries of the Mediterranean basin, and wine continues to appear on our tables by remaining a central element of our diet and culture. It should be noted that consumption is increasing in emerging countries (China, Brazil, Argentina) and in other industrialized countries (USA, Japan).

Also in this context, which combines popular beliefs, economic issues, and health issues both in terms of prevention with respect to the population at risk (i.e., the presence of alcohol), and in terms of its beneficial effects on health due to the presence of many bioactive molecules, it is important to provide a scientific rationale for moderate wine consumption in order to establish an effective public health policy and to identify new bioactive molecules in grapes that can have an advantage in a healthcare strategy.

During the last decade, numerous studies have revealed that a moderate consumption of wine, as part of a healthy diet, is associated with protective effects against relevant chronic diseases despite

its ethanol content and it harmful effects. For several years, numerous epidemiological studies have maintained that a moderate consumption of wine lowered the risks of mortality due to coronary diseases, compared to wine abstinence [1,2]. For example, in France, as compared with other western countries with a fat-containing diet, the strikingly low incidence of coronary heart diseases is partly attributed to the moderate consumption of red wine [3]. This is how what was commonly called the "French paradox" was born. Nevertheless, since the 1990s, this term has been controversial and seemed derogatory, the French paradox likely resulting more from a Mediterranean-type diet [4–6].

Indeed, several international long-term studies, sponsored by the World Health Organization (WHO), such as the MONICA (monitoring of trends and determinants in cardiovascular disease) study during the 1980s and more recently the PREDIMED (Prevención con Dieta Mediterrénea) Project, revealed the health benefits of wine that was associated with a diet rich in fruits, vegetables, and olive oil, commonly called the Mediterranean diet. The MONICA study has shown that in France, as compared with other western countries (such as the UK or the US), despite a fat-containing diet, a strikingly low incidence of coronary heart diseases was observed and is partly attributed to the moderate consumption of red wine [7–9]. The Mediterranean diet can be described as abundant in plant-based foods such as whole grains, legumes, seeds, fruits, and vegetables, with olive oil the main source of dietary fat; a limited intake of red and processed meat, favoring a low to moderate intake of low-fat dairy and a moderate consumption of fish; and emphasizing regular, but moderate, alcohol (mostly red wine) consumption with meals [10]. The Mediterranean diet has thus gained strong scientific support for providing protection against relevant chronic diseases such as cardiovascular diseases (CVDs), diabetes, as well as protection against some cancers [11,12]. These observations have aroused increasing interest in the scientific community in understanding the underlying mechanisms involved. In view of these epidemiological results, it seems essential to standardize studies to describe the effects resulting from a consumption of red wine alone. In this way, to study the potential effect of red wine on cardiovascular events, we have shown an amelioration of blood parameters (decrease in total cholesterol andlow density lipoprotein, LDL); increase in erythrocyte membrane fluidity and antioxidant status) in a group of selected post-myocardial infarct patients receiving 250 mL/day of red wine for 2 weeks, in comparison to patients receiving water in a controlled environment in the hospital [13]. For the first time in a controlled environment, these results reinforced the idea that a moderate consumption of red wine, even for a short period, associated with a "Western prudent" diet, improves various blood parameters in the lipid and antioxidative status in patients with previous coronary ischemic accidents.

Other studies tend to show beneficial effects related to wine consumption on the occurrence of degenerative pathologies, for example, age-related macular degeneration (AMD) [14], dementia [15–18], and cancers [19,20]. Subsequently, various studies have been conducted to determine the effect of different preparations enriched with wine polyphenol or polyphenol grape extracts on various pathologies such as cardiovascular [21,22], ocular [23], inflammatory and age-related degenerative diseases [24,25], and cancers [26,27] (Figure 1).

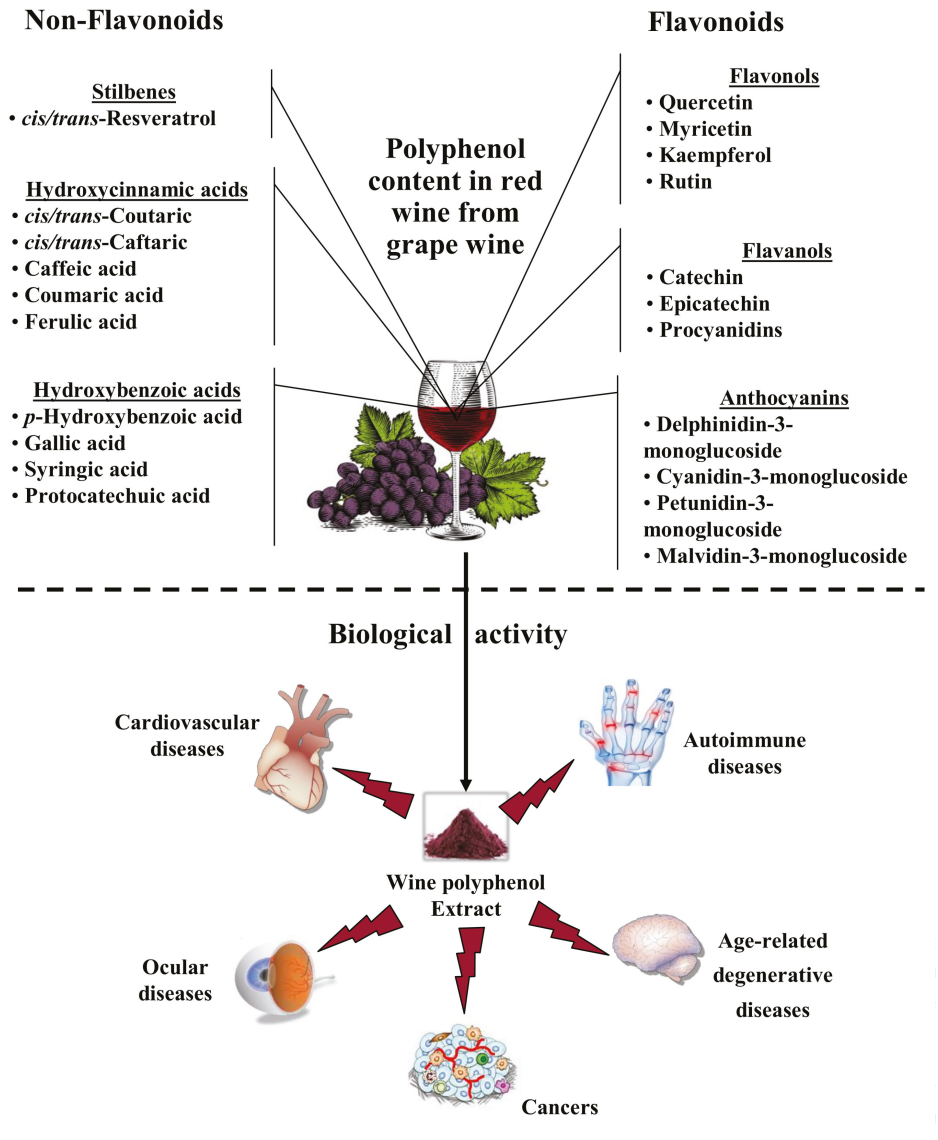

Figure 1. Major constituents in red wine from grapes and the potential biological effects against various diseases.

2. Wine and Cancers

2.1. Wine Composition and Variability of Effects

Cancer is one of the major causes of death in the world and is responsible for an estimated 9.6 million deaths in 2018. The most common cancers equally affect the lung and breast, with 2.09 million cases, colon (1.80 million cases), prostate (1.28 million cases), skin (non-melanoma) (1.04 million cases), and stomach (1.03 million cases). Tobacco use, alcohol abuse, an unhealthy diet, and physical inactivity are described as major cancer risk factors worldwide and modifying or avoiding these key risk factors can significantly reduce (30%–50%) the burden of cancer [28,29]. Therefore,

the strong interrelationship between nutrition and diet and the occurrence of tumors is abundantly described in the literature [30], and it is observed that a plant-rich diet may prevent 30%–40% of all cancer types [31–33]. Also, high intake of dietary fiber from fruit, vegetables, and whole grains is inversely associated with colorectal cancer risk [34]. Such benefits have been partly attributed to the presence of significant amounts of phenolic antioxidants in fruits and vegetables and are believed to contribute to their chemopreventive effects, notably in the colon [35–37]. Therefore, several studies (in vitro and/or in vivo) have demonstrated the anticancer potential of wine phenols (i.e., resveratrol, quercetin, (+)-catechin). In this way, various case–control studies have shown that a moderate red wine consumption exerts a protective effect on colorectal cancer in both men and women [19,20]. Moreover, other case–control studies have examined the association between wine and the Mediterranean diet, showing a lower risk of colon cancer and certain other cancers, such as urinary tract tumors, compared to other diets [38–40]. Nevertheless, one study did not find an inverse association between moderate red wine intake and the risk of colorectal cancer [41] or breast cancer [42]. This controversy may result from the amount and quality of polyphenols present in red wine. Indeed, red wine contains a range of biologically active polyphenols, including phenolic acids, trihydroxystilbenes, and flavonoids (Figure 1). In previous studies, we have shown both that a mixture of polyphenol extract from vine shoots demonstrates more antiproliferative activity on colon cancer cells than resveratrol alone, due to a synergism between polyphenols [43], and that the quantity and quality of the polyphenols present in wine also played an important role.

Wine composition is a complex and unique combination dependent on various factors such as the vine, the climate, the country, and the year. Thus, the amount of polyphenols in wine, although varying greatly, is estimated to be around 190–290 mg/L in white wines and 900–2500 mg/L in red wines [44,45]. This variability of the polyphenol composition seems very important in determining its effects. We demonstrated in a previous study that the lengthening of the maceration time modified and enriched the polyphenol composition of red wine [26]. In comparison with red wine extracts, whose maceration time was less and therefore whose polyphenol composition was lower, the extract resulting from a longer maceration in red wine showed a more pronounced antiproliferative effect, with respect to the colonic cancer lines tested [26]. The quantitative aspect is not the only important parameter; the qualitative composition of the wines is also a crucial factor in the observation of the beneficial effects or their absence. Very interestingly, some polyphenols do not act in a synergistic manner but rather in an additive manner and in some cases have an opposite effect [26]. These data raise the crucial role of the polyphenol composition of wine where an imbalance between polyphenolic species may increase or conversely reduce their beneficial effects. The presence of (+)-catechin reduces the synergism effect between resveratrol and quercetin, which could explain the differences studies have shown in colon cancer risk reduction with moderate red wine consumption in humans [19,20], or in animal models [27,46], while others showed no effect [41,47]. It therefore seems essential to study the effects of a wine in relation to its phenolic composition. Some of these polyphenols present in large quantities have a strong activity when they are studied separately. This is particularly the case for the most well-known resveratrol [48,49], quercetin [50], (+)-catechin [51], and gallic acid [52,53], which present a variety of chemopreventive properties.

2.2. Wine and Colorectal Cancer

Consequently, the effects of wine consumption, particularly in a healthy population, may depend on the composition of the wine or grape polyphenols and their bioavailability [54,55]. Otherwise, dietary polyphenols exert a beneficial effect at a local level (colon) directly, during their passage through the oral cavity and the gastrointestinal tract, and at a systemic level, after being absorbed. Therefore, one of the organs that can be a targeted is the intestine and colon.

Colorectal cancer (CRC) is the third most common form of cancer occurring worldwide. A total of 1.8 million cases each year are recorded [28]. Epidemiological and experimental studies have shown that the risk factors of developing colon cancer can be attributed mostly to multifactorial environmental

factors. Therefore, the majority (95%) of CRC diagnoses begin as noncancerous polyps of the intestinal epithelium on the inner lining of the colon or rectum that have accumulated oncogenic mutations over time. Noncancerous polyps may become malignant and transform into adenomatous polyps if left undetected. Progression through the various stages of the adenoma–carcinoma process is significantly influenced by environmental factors inherent in the western lifestyle, such as the diet, the sedentary lifestyle, as well as smoking and the consumption of alcohol. Indeed, the consumption of high levels of red meat and fat together with low levels of fruits and vegetables has been suggested to increase the risk of CRC [56]. Several studies indicate that the establishment of nutritional prevention could significantly reduce the occurrence of colon cancer. As documented in the literature, the benefits of the Mediterranean diet include protection against cardiovascular disease, metabolic diseases such as diabetes, obesity, and various cancers, and now is a recommended diet, among others, in strategies for cancer prevention. However, the mechanisms involved in the protection against CRC by patients following the Mediterranean diet are not completely identified and understood. For more insight on the Mediterranean diet, Donovan et al. have reviewed preclinical and clinical studies conducted over the last 10 years on the impact of a Mediterranean diet-eating pattern [57]. New cancer prevention strategies include a number of dietary constituents described as promising chemopreventive agents [58]. Chemoprevention is defined as the use of specific natural products or synthetic chemical agents to delay, prevent, or reverse lesions before the development of invasive cancer [59]. Otherwise, previous research has demonstrated that polyphenol-rich extracts from wines and grapes have the ability to modulate mutagenesis and prevent tumor initiation and promotion [46]. Gronbaek et al. demonstrated that regular and moderate consumption of red wine is associated with a 22% decreased risk of cancer [60]. Various reports have demonstrated the anticancer action of red wine polyphenols in animal models. For example, tumoral C26 growth was significantly reduced with red wine polyphenols in BALB/c mice [27]. In this study, red wine extract (RWE) decreased tumor vascularization and the expression of proangiogenic factors including vascular endothelial growth factor (VEGF), metalloproteinases (MMP-2, MMP-9), and cyclo-oxygenase-2 (COX-2) proteins (Figure 2). The inhibition of cell proliferation with the expression of p21, an inhibitor of the cell cycle, and the expression of tumor suppressor genes, including p16INK4A, p53, and p73, was observed. RWE is able to act on the development of colorectal cancer through the prevention of aberrant crypt foci (ACF) (Figure 2). In a preclinical study on male CF-1 mice developing intestine polyp preneoplasia with azoxymethane (AOM) injection [61], we showed that mice receiving red wine extract in the diet, at a concentration of 500 mg/kg for 6 weeks, reduced the total number of AFCs per centimeter of colon as compared to the control group [26]. This result is interesting because the lower part of the colon is often associated with rectal cancer. Very recently, this study was reinforced by a report showing that dried red wine, pomegranate extract, and α-tocopherol added at one dose to cured meat, and the withdrawal of sodium erythorbate, significantly decreased the number of precancerous lesions (mucin-depleted foci, MDF) per colon in rats [62]. However, white grape and rosemary extracts, which do not have the same polyphenol composition, did not. The molecular mechanism involved seems to be the suppression of fecal excretion of nitrosyl iron [62].

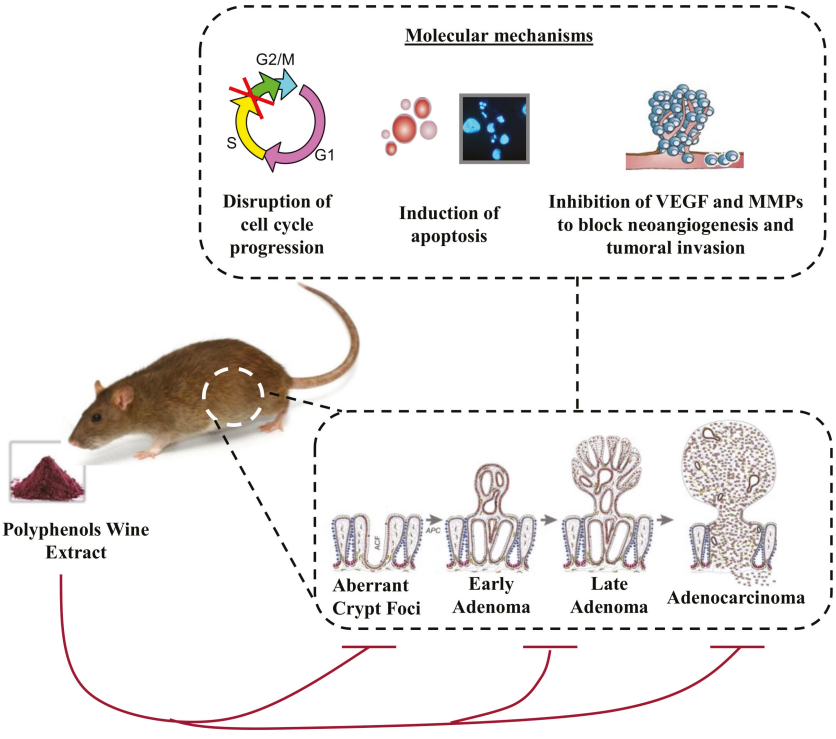

Figure 2. Wine polyphenol extract is able to prevent aberrant crypt foci formation in various animal models (mice, rats), which is the first step of colorectal carcinogenesis, and can block the different steps leading to adenocarcinoma development. The effects involved different molecular mechanisms such as an arrest of the cell cycle in the S phase, an induction of apoptosis through caspase activation, and an inhibition of angiogenesis and tumoral invasion through a decrease in vascular endothelial growth factor (VEGF) secretion and matrix metalloproteinases (MMP) activities.

Other targets that have been thoroughly described in the occurrence of colorectal cancer are the enzymes involved in arachidonic acid metabolism (i.e., cyclooxygenase and lipoxygenase). The latter is inhibited in the presence of grape seed extract or red wine polyphenolic compounds in various colorectal cancer cell lines or hepatocarcinoma cells [63].

Therefore, the present findings provide in vivo evidence for the antiangiogenic, antiproliferative, and proapoptotic effects of red wine polyphenols, associated with an effective inhibition of colon carcinoma tumor growth in mice. Red wine polyphenols target several key processes for tumorigenesis, supporting their role as potential chemopreventive agents against cancer.

2.3. Wine and Prostate Cancer

Prostate cancer (PCa) is the fourth most common form of cancer occurring worldwide. A total of 1.28 million cases each year are recorded [28]. There are well-established risk factors for PCa, such as family history [64,65], hereditary genes [66], racial and ethnic background, and age. Also, a wide variety of exogenous, environmental, and lifestyle factors have been shown to impact the risk of PCa development and progression. A study has shown a significant dose–response relationship between the level of alcohol intake and the risk of PCa [67]. Despite an association between alcohol intake and PCa risk, the effect of wine consumption on PCa risk is not yet fully understood.

According to the meta-analysis undertaken by Vartolomei et al. on 17 studies (611,169 subjects), there is an antagonist effect, such that moderate white wine consumption increases the risk of PCa, whereas moderate red wine consumption had a protective role against PCa [68]. This difference can be explained by the anticarcinogenic effect of polyphenols that are mainly found in red wine, which may balance any other negative or harmful effects, and by the bioactivity of the polyphenols present in red wine.

A recent study with a grape skin extract (MSKE), which is derived from muscadine red grapes, highlights a molecular mechanism involving an activation of unfolded protein response (UPR)-mediated autophagy and the subsequently mediated apoptosis [69]. The existing cross-talk between autophagy and apoptosis has been considered a key factor in the development and treatment of cancer [70]. MSKE induced apoptosis via the up-regulation of endoplasmic reticulum (ER) stress-driven caspase-3, -7, and -12. MSKE also prompted the down-regulation of antiapoptotic and survival proteins, such as annexin A4 (ANXA4), a member of the Ca^{2+}-regulated and phospholipid-binding annexin superfamily, which is regularly increased in many cancer types [71–73].

Colorectal and prostate cancers are not the only ones that can be affected by the action of wine polyphenol extracts; many other studies have shown an induction of apoptosis and a modulation of the protein that controls either oxidative stress or the cell cycle on many models, such as breast cancer [74] and leukemias [75].

In a similar manner to the different compounds that compose it, the wine extracts can modulate the activation of the kinase cascades induced by many activators. Resveratrol, a major phenolic component, can inhibit the mitogen-activated protein kinase (MAPK) pathway activation, which is mediated by various promoters [76,77]. For example, in vitro, resveratrol inhibits phorbol-12-myristate-13-acetate (PMA)-mediated activation of c-Jun N-terminal kinase (JNK) [78]. In vivo, pretreatment of the dorsal skin of female mice with resveratrol decreases the phosphorylation of extracellular signal-regulated protein kinase (ERK), as well as the catalytic activity of ERK and p38 MAPK, which are stimulated by various stimuli [77,79,80]. Lee et al. reported that red wine extract inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation of JB6 promotion-sensitive mouse skin epidermal cells. The activation of activator protein-1 (AP1) and nuclear factor-kappa B (NFκB), induced by TPA, was dose-dependently inhibited by RWE treatment [81]. Consequently, by its blocking action on the stimulus-mediated MAPK pathway activation, red wine extract could possess an antitumor-promoting property.

2.4. Other Beneficial Properties of Wine in Cancer

These polyphenol extracts could both serve as sources of new bioactive compounds and also reduce the deleterious effects of certain chemotherapies. A study on various cancers has shown that a red wine extract lowered the side-effect toxicity of cisplatin treatment [82]. In the same way, a grape seed extract has been shown to protect intestinal epithelial cells prior to damage induced by 5-fluorouracil (5-FU) in female Dark Agouti rats [81]. These females were gavaged with 1 mL of polyphenol extract (400 mg/kg) daily (day three to 11), and received 5-FU (150 mg/kg) by intraperitoneal injection on day nine to induce mucositis. Compared with 5-FU controls, polyphenol extract significantly reduced myeloperoxidase activity in the proximal jejunum and distal ileum, decreased qualitative histological scores of damage in the proximal jejunum, increased villus height in the proximal jejunum and distal ileum, and attenuated the 5-FU-induced reduction of mucosal thickness by 16% in the jejunum and 45% in the ileum [81].

More interestingly, red wine extract can present an additive effect, when combined with cyclophosphamide or with cisplatin, to inhibit the tumor growth in mice with Ca755 mammary carcinoma or with Guerin carcinoma [82].

In view of these results, many teams and firms then made lyophilized extracts enriched with polyphenols from grapevines. For example, Liofenol™ contains natural Gocciorosso red wine lyophilized extracts, is devoid of alcohol, and is composed of a variety of components, such as polyphenols [83]. This preparation reduces colon cancer cells with an increase of p53 and p21 protein

expression. Moreover, the authors observed a strong induction of antioxidant response, with the activation of the transcriptional factor Nrf2, involved in redox homeostasis and differentiation, without altering tumor sensitivity to chemotherapy with Tam and etoposide [83].

3. Conclusions

Moderate wine consumption, part of the Mediterranean diet, has been associated with potential protective effects, not only on cardiovascular pathologies but also on several cancer types. These protective effects have been attributed to wine microconstituents, such as polyphenols, among which resveratrol has been the most widely studied so far. However, much effort has been extended to characterize and understand the complex composition of wines, in particular red wine, whose polyphenol content is highly dependent on winemaking processes, the type of vine, climate, country, and the age of the wine. We have notably provided evidence that the efficacy of red wine extracts in inhibiting colon cancer cell line proliferation depended on their polyphenolic content and composition. Indeed, taken individually, red wine polyphenols have been shown to limit several stages of tumorigenesis, supporting their chemopreventive properties; however, some polyphenols may have antagonist activities, thus limiting their beneficial effects. These aspects are of importance as not all preclinical trials in humans have shown a positive effect of wine extract consumption on the occurrence of cancers, which could be partly explained by the dose used and the composition of the extracts. Nonetheless, a great deal of evidence supports the bioactivity of red wine extracts in various cancer type models, both in vitro and in vivo, in animals. Compared to individual red wine chemical constituents, RWE has been able to target the most commonly deregulated signaling pathways in cancer and prevent some of the side effects of conventional chemotherapies. Hence, diet supplementation with RWE, which does not seem to present any toxicity, at least in animal models, would be valuable in chemoprevention strategies; however, much work remains to be done to discover how best to use it (e.g., identification of the most efficient combination of microcomponents for synergistic effects), which may benefit from taking into account individual genetic variability and metabolism.

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diseases

MDPI

Review

Is a Meal without Wine Good for Health?

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Abstract: Hippocrates, the father of medicine, had said: "Wine is a thing wonderfully appropriate to man if, in health as in disease, it is administered with appropriate and just measure according to the individual constitution." Wine has always accompanied humanity, for religion or for health. Christians and Jews need wine for the liturgy. For Plato, wine was an indispensable element in society and the most important in the symposium. In this second part of the banquet, mixed with water, the wine gave the word. If the French paradox made a lot of ink flow; it was the wine that was originally responsible for it. Many researchers have tried to study alcohol and polyphenols in wine, in order to solve the mystery. Beyond its cardiovascular effects, there are also effects on longevity, metabolism, cancer prevention, and neuroprotection, and the list goes on. The purpose of this work is to make an analysis of the current knowledge on the subject. Indeed, if the paradigm of antioxidants is seductive, it is perhaps by their prooxidant effect that the polyphenols act, by an epigenetic process mediated by nrf2. Wine is a preserve of antioxidants for the winter and it is by this property that the wine acts, in an alcoholic solution. A wine without alcohol is pure heresy. Wine is the elixir that by design, over millennials, has acted as a pharmacopeia that enabled man to heal and prosper on the planet. From Alvise Cornaro to Serge Renaud, nutrition was the key to health and longevity, whether the Cretan or Okinawa diet, it is the small dose of alcohol (wine or sake) that allows the bioavailability of polyphenols. Moderate drinking gives a protection for diseases and a longevity potential. In conclusion, let us drink fewer, but drink better, to live older.

Keywords: wine; Mediterranean diet; Okinawa diet; health; nrf2; alcohol; polyphenols; hormesis; cardiovascular protection; cancer; Alzheimer; metabolic disease

1. Introduction

The French paradox, a concept described by Serge Renaud, describes the observation that, in France, despite a high consumption of saturated fats, a low cardiovascular mortality rate is described, compared to other "industrialized" countries that consume the same type of food.

The explanation of this French paradox consists of a moderate consumption of wine during meals. There is also a north-south gradient, with an even lower cardiovascular mortality rate in Toulouse (consumption of red wine, olive oil, and duck fat) as compared to Lille, where meals are more based on saturated fats and where the favorite drink is beer.

Although the consumption of red wine is decreasing, the eating habits of adults are changing towards a Mediterranean-style meals and wine remains a social link. The risk of considering wine as just another spirit, is that our young people no longer seek the pleasure of a conversation around a good meal and a good bottle, but look for an immediate euphoric effect, such as in binge-drinking or the holiday heart syndrome. It would be necessary to follow a policy of educating young people so that they turn to wine, rather than a premix or other strong alcohol, to rediscover the pleasure of a reasonable consumption of wine which does not promote addiction.

We know the protective power of a regular and moderate consumption of red wine, in terms of primary and secondary prevention, and prevention of cancer (particularly studied for resveratrol).

We are now interested in its effects on aging and, in particular, its protective role on the occurrence of dementia.

The ideal dose seems to be two to three standard drinks a day. This is already what St. Benedict recommended for his monks, in his rule number 40 [1]. The proposed amount was one hemin a day, which corresponds to three glasses a day. On the other hand, this did not oblige the monks to abstain, but specified that the fat monks could take a little more. The red wine was, back then, the only source of antioxidants for the winter, as canning and freezing did not exist. It is well established that alcohol, in a glass of wine, improves the bioavailability of polyphenols in the food bolus. This is the principle of the Cretan diet. It is also one of the factors that make the Japanese diet beneficial, in addition to its richness in omega 3 polyphenols and antioxidants (tea catechins, ginger, wasabi, etc.). History has shown that our civilization has always been closely linked to wine. Vinification allows the extraction and the conservation of the antioxidants, thanks to the alcohol, and it is still the wine's alcohol that allows our body to absorb the antioxidant polyphenols that are beneficial to our health [2]. Why demonize this product, which has long been one of the only effective products of our former pharmacopoeia?

2. Free Radicals and Antioxidant Defense

Reactive Oxygen Species (ROS) can be exogenous and endogenous. Exposure to pollution, prolonged sunlight, absorption of drugs, alcohol, and smoking causes ROS production that can outperform the endogenous antioxidant defenses. Unfortunately, there are not enough fruits and vegetables in the food consumed by people (polyphenols, vitamin C, vitamin E, and carotenoids) that can boost the endogenous antioxidant defenses.

All cells, by their metabolism, produce small amounts of derivative oxygen reagents. Indeed 1 to 2% of the oxygen present is diverted to form free radicals. ROS are mainly produced at the mitochondrial level during the process of conversion of oxygen into water, producing a superoxide radical (O^{2-}). This superoxide radical can also be produced at the microsomal and plasma levels, by Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases. The superoxide radical is converted into hydrogen peroxide (H_2O_2) (more stable) by Superoxide Dismutase (SOD), and then into water, either by catalase (CAT) or glutathione peroxidase (GPx) [3]. ROS can react with different biological substrates, such as lipids, proteins, and DNA. Oxidative stress is the balance–imbalance of prooxidant and antioxidant compounds. ROS are involved in the expression and regulation of the functions of cell proliferation and cell death. The study of various pathologies, such as neurodegenerative diseases, atherosclerosis, and cancer, has shown that ROS also act as inflammatory mediators. ROS are very unstable, and their lives are very short (10^{-4} seconds). Their reactivity lies in their search for an electron to match their single electron. $O_2\bullet^-$ and hydrogen peroxide (H_2O_2) are not very active. $O_2\bullet^-$ can capture H^+ to give $HO_2\bullet$, which would be the reactive form of $O_2\bullet^-$, that is capable of initiating a lipid peroxidation. $O_2\bullet^-$ can also be dismuted to H_2O_2 and O_2 (spontaneous reaction or catalyzed by superoxide dismutase), react with NO• to form the peroxynitrite anion $ONOO^-$, a powerful oxidant, or reduce the transition metal ions. O_2 is produced, in particular, by the reduction of molecular oxygen in mitochondria by NADPH oxidase or by xanthine oxidase, an enzyme of the purine metabolism. The hydroxyl radical HO• is one of the most oxidizing chemical species and can very quickly attack most biological molecules. HO• is produced by the reduction of H_2O_2 by low-valence metal ions, such as Fe^{2+} or Cu^{2+}, free or complexed (heme); the Fenton reaction (Figure 1).

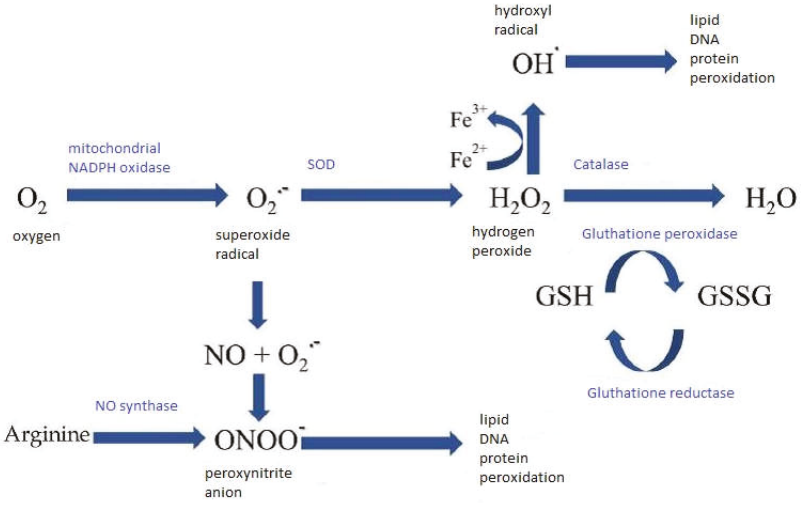

Figure 1. Production of Reactive Oxygen Species.

Mitochondria are the essential organelles responsible for the production of energy, in the form of ATP, which is necessary for the cell function. The respiratory chain is a permanent source of ROS. Complexes I and III are the preferred sites for the ROS production [4–6].

Expression of nuclear genes encoding mitochondrial proteins, as well as mtDNA replication and transcription mechanisms, are regulated primarily by transcription factors and transcriptional coactivators.

2.1. Transcriptional Factors Nrf2 (Nuclear Respiratory Factor 2)

Nrf2 is an important transcription factor that protects the mitochondria from stress oxidants by inducing anti-oxidant and detoxification genes, by its binding to the Antioxidant Response (ARE). However, it would have a facilitating role on the formation of atheroma. The Keap1 protein binds to the Nrf2 protein to inhibit it. In the quiescent state, Nrf2 is anchored in the cytoplasm by binding to Kelp1 protein (Kelch-like ECH-associated protein 1), which facilitates the ubiquitination and proteolysis of Nrf2. This mechanism contributes to the repressor effect of Keap1 on Nrf2. Activation of Nrf2 leads to a coordinated antioxidant and anti-inflammatory response [7–12].

2.2. Antioxidant Defenses

2.2.1. Enzymatic Defenses Systems

Enzymatic systems are the most important cellular defense systems to control oxidative attacks. They protect or detoxify the body against ROS.

Superoxide dismutases (SOD) and catalase (CAT) play a protective role, while glutathione peroxidases (GPx) play a role in detoxifying the ROS. There are three SODs: That located in the cytoplasm involving copper and zinc as co-factors, named Cu^{2+}/Zn^{2+}-SOD (SOD-1); that located in the mitochondria which has manganese as a co-factor, Mn^{2+}-SOD (SOD-2); and the secreted extracellular Cu^{2+}/Zn^{2+}-SOD (SOD-3). SODs are metalloenzymes that catalyze the disproportionation of superoxide anions into hydrogen peroxide and oxygen, ten thousand times faster than the spontaneous disproportionation of the superoxide anion. The reduction of H_2O_2 in the cytosol will depend on the Cu^{2+}/Zn^{2+}-SOD and on glutathione peroxidase (GPx). Catalase is localized in the mitochondria and peroxisomes, it can also reduce H_2O_2, but due to its low affinity for this radical, GPx is more efficient [13]. On the other hand, an excess of H_2O_2 or the presence of transition metals,

Fe^{2+} and Cu^{2+}, can lead to the formation of the hydroxyl radical (OH) and nitro reactive metabolites. GPx detoxify hydrogen peroxide and lipid peroxides by coupling their reduction to oxidation of a reducing substrate, glutathione [14]. GPx are seleno-dependent enzymes that contain four selenium atoms in the active center of the enzyme. Thus, a selenium deficiency causes a decrease in the GPx activity, while an abundance restores it. Two forms of GPx differ from each other in structure and activity [15,16].

2.2.2. Non-Enzymatic Antioxidant Systems

Non-enzymatic antioxidants may be endogenous water-soluble agents (glutathione, uric acid, bilirubin, and ubiquinol (coenzyme Q10)) or be provided by the diet (vitamin C and E, carotenoids, polyphenols) [17–19]. Glutathione (GSH) is an endogenous water-soluble agent that has important antioxidant properties. Glutathione prevents the oxidation of thiol groups, thanks to its reducing power. The regeneration of the GSH thiol function from the oxidized form is done through the activity of the glutathione reductase (GR). It can chelate the cuprous ions and, thus, limit their participation in the Fenton reaction. In addition, it is directly involved in the repair of the oxidative damage to DNA. Water soluble vitamin C can behave as an antioxidant or prooxidant, depending on the dose used. Too high a dose of ascorbic acid becomes toxic to the body. Ascorbic acid can take a reduced or oxidized form, depending on the pH of the medium in which it is and the presence of transition metals. The passage from one form to another is affected by glutathione/glutathione reductase and it generates an ascorbyl radical. Thus, it is considered as a redox couple with an ascorbyl radical, capable of capturing the ROS and the singlet oxygen. Vitamin E exists in eight natural forms, four tocopherols and four tocotrienols. Tocopherol is liposoluble and has the capacity to capture and stabilize the single electron of free radicals, following the reaction:

$$\text{tocopherol-OH} + \text{LOO}\bullet \rightarrow \text{tocopherol-O}\bullet + \text{LOOH (LOO}\bullet\text{: free radical lipid)}$$

The radical-bearing tocopherol may react with a new free radical to form a neutral species, or be regenerated by vitamin C, glutathione, or the coenzyme Q10. Vitamin E mainly plays its role as an antioxidant in biological membranes. Mitochondria, which generates free radicals, contain high levels of vitamin E in their lipid membrane, consisting of polyunsaturated fatty acids and are subjected to oxidative stress.

Natural polyphenols include a broad set of chemical substances, comprising at least one aromatic nucleus, bearing one or more hydroxyl groups, in addition to other components. They can range from simple molecules, such as phenolic acids (gallic acid), to highly polymerized compounds of more than thirty thousand daltons, such as tannins (tannic acid). Polyphenols are commonly subdivided into simple phenols, phenolic acids (derived from benzoic or cinnamic acid), stilbenoids (two C6 rings linked by two carbon atoms), flavonoids, isoflavonoids and anthocyanins, and condensed tannins (Figure 2).

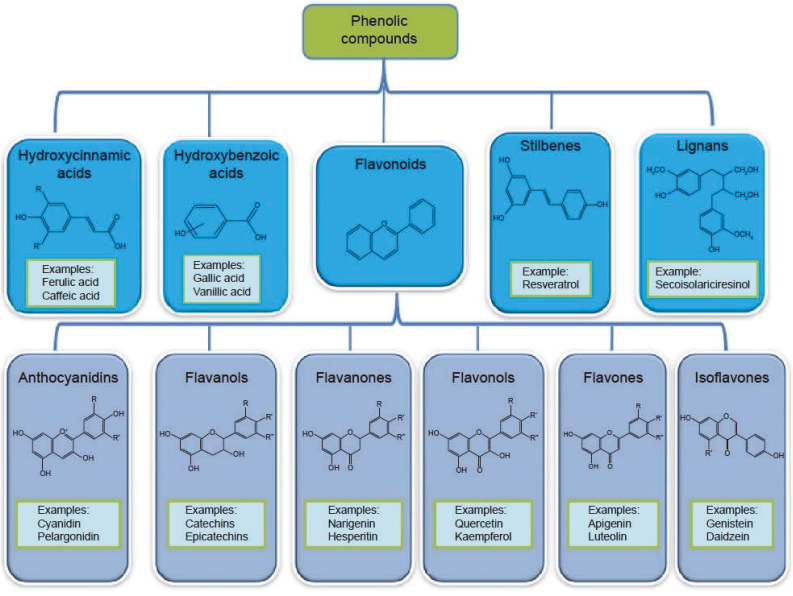

Figure 2. Phenolic Compounds.

The fruits and vegetables consumed provide more than eight thousand polyphenols. Flavonoids are the most abundant polyphenols in our diet and over four thousand have been identified [20]. Flavonoids have a common C6-C3-C6 structure. Two aromatic rings (A and B) are linked by a chain of three carbons forming an oxygenated heterocycle (C) [21,22] (Figure 3).

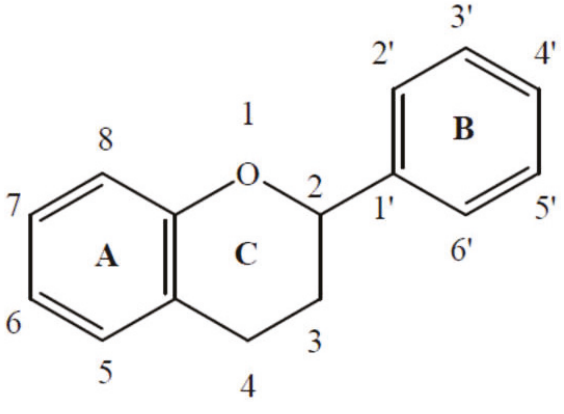

Figure 3. Flavonoids structure.

Among the flavonoids, flavanones are responsible for the bitterness of grapefruit. The tannins are responsible for the astringency of various fruits (skin and grape seeds) and anthocyanins, the color of red fruits. Polyphenols are present in various natural substances, in the form of anthocyanin in red berries and red wine, as proanthocyanidines in chocolate and wine, as coffee-quinoline and feruloylquinic acids in coffee, flavonoids in citrus fruit, and in the form of catechins, such as epigallocatechin gallate in green tea, quercetin in apples, onions, and red wine. These basic carbon skeletons are derived from the secondary metabolism of plants, developed by the shikimate

pathway [23]. Shikimic acid is an important biochemical intermediate in plants and microorganisms. It was isolated for the first time in 1885 by the Dutchman Johann Frederik Eijkmann, from the flower of shikimi (illicium anisatum or Japanese badian). Shikimic acid is the precursor of phenylalanine and tyrosine, aromatic amino acids; indole, indole derivatives and tryptophan; many alkaloids and other aromatic metabolites, such as resveratrol; tannins; lignin; and salicylic acid.

Polyphenols in wine are present in the film and seed. In the roundup, tannins are often undesirable, from a taste point of view, and maceration is most often performed on the broken-up grapes. The solubilization of these polyphenols takes place during the alcoholic fermentation, it increases with the alcoholic degree of the grape must. Maceration is the stage of winemaking that extracts the phenolic compounds. The condensed tannins are oligomers or polymers of flavanols. They consist of flavan-3-ols units, linked together by carbon-carbon bonds. The conformations are helicoidal. The passage in barrels (breeding) allows a micro oxygenation of the wine, through the pores of the wood, and the polymerization of the tannins. The barrel provides little or no wood polyphenols. It is this polymerization of tannins by microoxidation, which confers on them their anti-oxidant properties. This same phenomenon is used for tea—microoxidation by successive passages of the tea through glass teapots, among the Arabs, or microoxidation with the tea whip macha (chasen), among the Japanese (Figure 4).

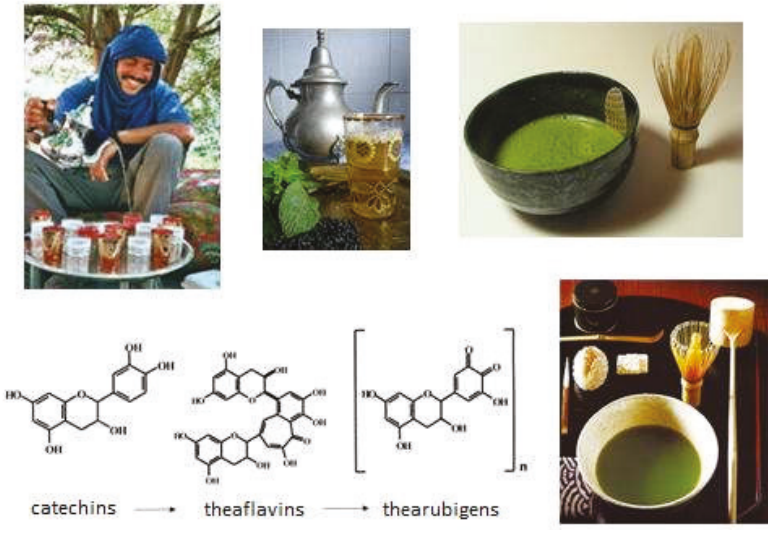

Figure 4. Polymerization of catechins.

3. Wine and Cardiovascular Protection: The French Paradox

Many studies have tried to unravel the "French Paradox", a term used by Serge Renaud in 1991 on CBS. This paradox consists of a low mortality of cardiovascular origin, despite a high consumption of saturated fats [13,24]. The epidemiological studies of Saint Leger [25] and Keys [14] (thirteen thousand subjects followed for twenty years, beginning in 1952) showed that the Mediterranean basin, and more particularly Crete, was protected, probably because of a specific diet. The MONICA project [26] confirmed the particular position of France, and showed a south-north mortality gradient, confirming the probable origin of the difference in diet between Toulouse and Lille. The intake of mono and polyunsaturated fats, garlic, duck fat, low-meat diet, high intake of fruits and vegetables, have been advanced, to identify the Mediterranean diet; the Cretan diet is also characterized by this frugality. All Mediterranean civilizations are also wine civilizations, and some wanted to study an important or even essential element of this diet that is protective against cardiovascular diseases. The antioxidant

effect of red wine flavonoids appears to be one of the mechanisms of vascular protection provided by the Mediterranean diet. The term "French paradox" describes the paradoxical situation in which the French population, more particularly the southern one, dies less of cardiovascular pathology than northern Europeans and Americans, despite high and comparable risk factors (smoking, rich diet including saturated fats, sedentary lifestyle). Given the lack of a fundamental difference in the diets of the different populations studied, Serge Renaud spoke of the role of red wine consumption, because the French consume 89 liters of red wine a year against 7.7 for the British [27]. The French paradox was then attributed to the consumption of wine because the French are the largest consumers of wine in the world (after the Luxembourgers) [28]. French gastronomy is reputed to be rich in lipids, especially in saturated fatty acids, with more than four hundred cheeses, charcuterie, mayonnaise, butter, foie gras, etc., in which it does not differ much from American or British food. The Mediterranean-type diet only concerns the South of our territory, that is to say 20% of the French [29]. The French, compared to other Western countries, rarely eat alone and prefer conviviality (work colleagues, family, friends, and neighbors).

If, as in all developed countries, the French mostly frequent supermarkets, they also do not neglect the small shops (bakeries, fishmongers, butchers, grocers, and markets) thus, favoring quality over quantity, in contrast to English people who are more likely to buy their groceries in bulk, during one visit to a supermarket, which already suggests more energy spent on supplies [30]. The French take more time to cook their dishes. They take more time to eat without consuming more. A discussion usually animates the meal. The gourmand/gourmet distinction is not so clear among the Anglo-Saxons. "Being gourmet" is more valued socially in France, than elsewhere. According to the American Time Use Survey (ATUS) [31], the average length of a meal in France is 15 minutes at breakfast, 38 minutes at noon, and 40 minutes at night, for a total of 93 minutes per day at the table, which is 30 minutes longer than the time spent by the Americans. The prolonged average duration of meals has a beneficial effect on the absorption and metabolism of fats, as well as on the peak-level of insulin secretion. The French eat less, between meals, to better appreciate the quality of their cooking (7.5% of our daily calories against 21% for Americans). Contemporary reality shows, such as "top chef" or "almost perfect dinner" add value to our national culinary expertise, on a daily basis. Two-thirds of French people eat at home at noon [32] and do not consume frequent snacks—only 6% consume fast food more than once a week [33]. We eat less red meat, and more cheese and yogurt than whole milk. The Southern French consume more olive oil while those in the North prefer butter. Only 10.2% of French people consume the five fruits and vegetables recommended by WHO. Thirty percent of the French people garden and grow a vegetable garden, which is unusual in Northern Europe. Vegetables are rather consumed fresh, not packaged industrially, and are often raw. The French is known to be a great consumer of garlic and snails. Seventy percent of the French people consume coffee every day. Ruidavets showed in 2004 that wine consumption in France has a positive impact on the quality of diet, compared to that of abstainers [34].

Generally, the midday meals are varied with successively an appetizer, a main meal, a cheese and then a dessert. The French eat smaller shares than Americans [35], which may already partly explain the French paradox. Montaigne was already talking about French alcohol consumption [36]. It is daily, moderate, and during meals, and conviviality is the most important. It differs from that of other Western countries. In France, drunkenness is a consequence, rarely a goal (except for alcoholics) it is the spreading of a pleasure and not the necessary cause of an intention of overflow. This mode of consumption is beneficial in many ways. Wine is also associated with relaxation, with the pleasure of conviviality. We know from Yusuf's meta-analysis [37] that moderate alcohol consumption (two drinks a day) is a cardiovascular protective factor, just like that of decrease in LDL, cessation of smoking, consumption of fruits and vegetables, and engagement in physical activity. Atheroma plaque in the coronary arteries is responsible for myocardial infarction and in the carotid artery it causes an ischemic stroke. The mechanism of plaque formation is complex and multifactorial, but begins with an accumulation of oxidized LDL in the intima of the artery. The effect of red wine on the oxidation of

LDL could be the key to the French Paradox. Indeed, the decrease of LDL (bad cholesterol) and an increase in the antioxidant power of the serum are two major protective factors. If there is less LDL and they are less oxidized, they will be deposited less in the arteries, so the formation of atheroma plaque will be delayed. The InterStroke study [38] confirms this data for stroke, at doses of one drink per day. Some will make the short cut by saying that a glass for the brain and two for the heart, which makes the three glasses allowed per day, since Saint Benedict. However, before recommending red wine as a beneficial dietary adjunct, it should be noted that the dose of alcohol must remain minimal so as not to cause the known deleterious effects of ethanol. On the other hand, it is certain that, whatever the red wine, it is a soup of antioxidants that can be preserved for a very long time when vinification is carried out in a traditional way (control of temperatures, long maceration, aging in new barrels), which is easily assimilated by the body because of the alcohol [39]. Alcohol provides superior bioavailability. It is not by chance that wine has always been part of the diet of the Mediterranean countries, it was the only way to conserve antioxidants (plants) for the winter. At a time of globalization, canning and freezing, we still have the fact that a glass of red wine brings much more antioxidants easily assimilated than large quantities of fruits and vegetables. The J curve represents the total mortality related to the number of glasses of wine, drunk per day. In Ellison's version [40] (Figure 5), the ischemic heart disease is isolated from other causes of mortality, if we notice an increase in deaths from accidents, cancers, or sudden death, for a daily consumption of more than three glasses, we notice a cardiovascular protection activity beyond these doses.

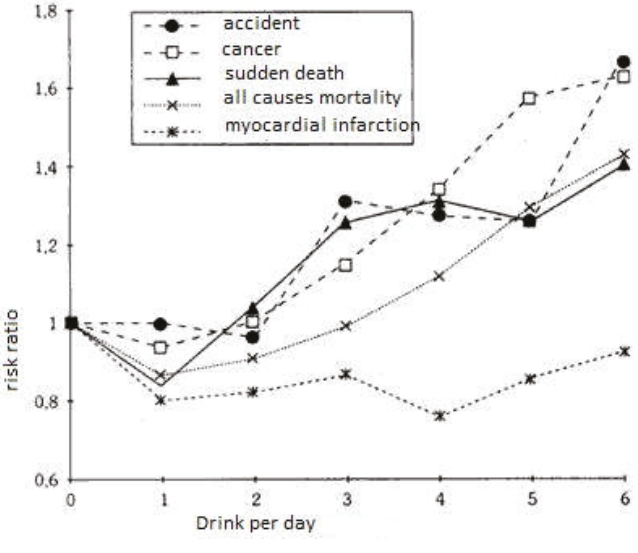

Figure 5. J curve [40].

A study published in 1997, showed that regular consumption of moderate doses of wine (200 mL) allows an antioxidant protection (Figure 6). All subjects were in good health (7 men and 3 women). The result was not significantly effective for all the wines. Only one wine had a speed effect on redox blood status (red 2). However, we can see that the antioxidant status was higher after the last wine. Therefore, we can suspect that the regularity of ingestion is the secret for a healthy consumption [41]. All of the tested wines were great wines of bordeaux and burgundy (red 2 was a bottle of Echezeaux of Domaine de la Romanee conti).

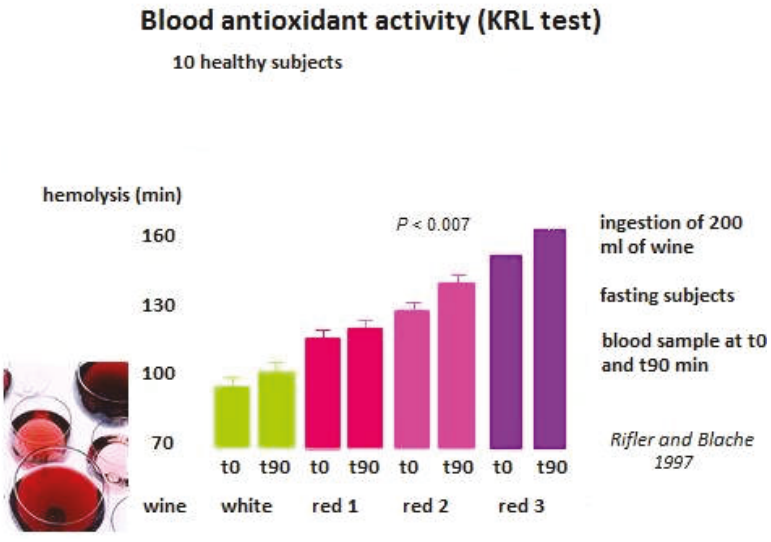

Figure 6. Blood antioxidant activity in healthy subjects drinking wine.

In another 2006 study, a supplement of 200 mL of red wine, during lunch, of healthy volunteers, showed a decrease in Total cholesterol and LDL cholesterol in just one week [41] (Figure 7). This regular and moderate consumption of red wine makes it possible to counteract the initiating factors of the atheromatous plaque, and have a cardiac protective effect in the primary prevention.

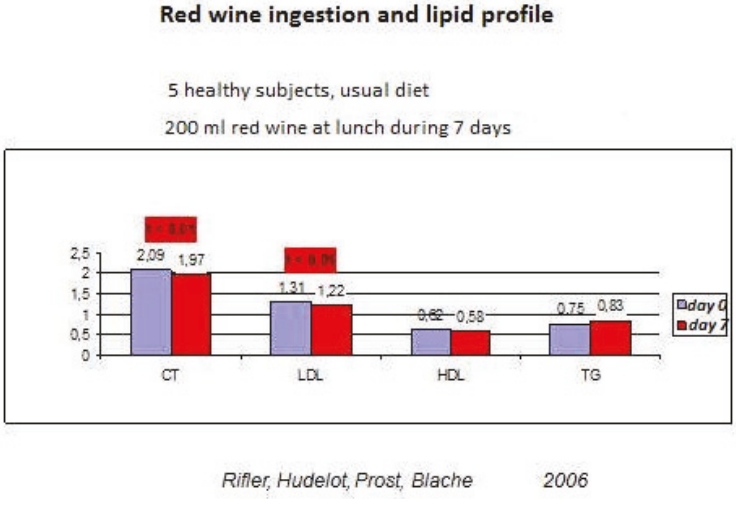

Figure 7. Lipid profile after 200 mL red wine ingestion at lunch. CT: Total cholesterol. LDL: Low density lipoprotein. HDL: High density lipoprotein. TG: Triacyl glycerol.

In the secondary prevention, we demonstrated in 2012 [42,43], the same phenomenon on patients with myocardial infarction, three days after the acute accident, a healthy diet was set up, with a drinking group of water, and the others drinking red wine (2 glasses a day). The wine group has demonstrated the same benefits as the primary prevention. The aim of this study was to study the effect of moderate and regular wine consumption on relevant biological parameters (lipid balance, measurement of serum

total antiradical resistance, level of fluidity of the red blood cell membrane) in patients in a situation of secondary prevention of a cardiovascular event, during their hospitalization for cardiovascular rehabilitation. Two similar populations were distinguished only by the consumption of wine. Thus, wine was the only hygieno-dietary parameter differentiating the two groups. The dietary diet chosen "Western prudent", was based on the dietary principles of the Lyon study, which is the reference in terms of cardiovascular protection, because of its resemblance to the Mediterranean diet. It consisted of a limited intake of saturated fats (animal fats, oils), a daily intake of fruits and vegetables, butter is replaced by olive oil and rapeseed, and limitation of cheese intake (2–3 times a week). The wine was made from the Pinot Noir grape variety, Villars Fontaine Haute Côte de Nuit, vintage 1999, with a high content of phenolic compounds (around 4000 mg/L of gallic acid equivalent). The wine was served without a label and without other indications. The consumption of wine was done exclusively during meals (1 glass at lunch, 1 glass at dinner). The anti-radical defense system is so complex that no specific test can provide an overall assessment of an individual's resistance to the attack of free radicals. The KRL® test used (Figure 8), was initially developed by the Spiral/Kirial International laboratory, at the request of NASA in 1987 [44]; it is a global measure of the antiradical defense potential. The KRL test makes it possible to evaluate the overall resistance against the aggression of free radicals. Thus, it is hoped that the higher the level of KRL, the less the individual will subject his arteries to the process of atherogenesis.

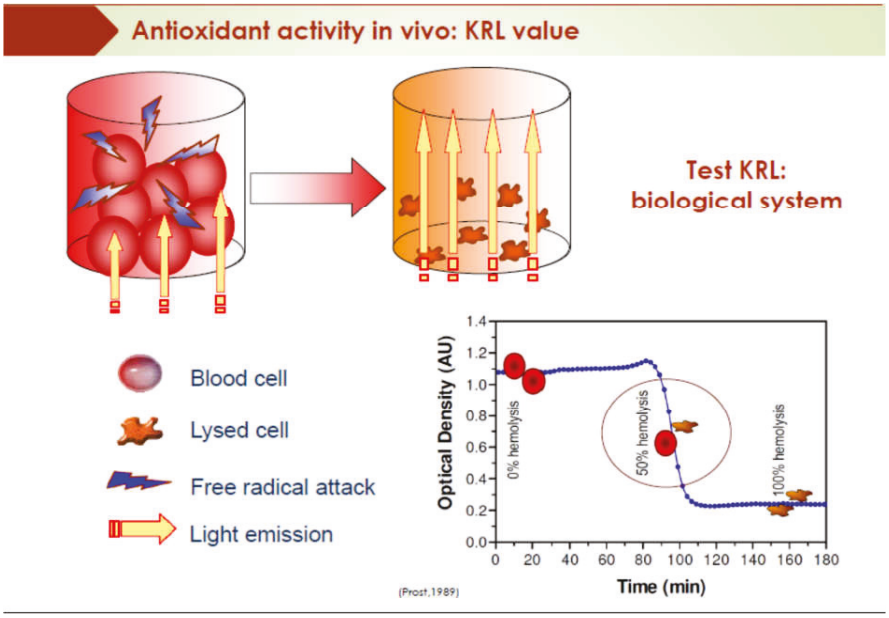

Figure 8. KRL test.

The blood sample is subjected to a standardized and reproducible aggression, induced by a generator of free radicals. The enzymatic and chemical defense systems allowing the integrity of the red blood cells are tested until cell lysis is obtained. The time after which 50% of the blood cells are lysed (half-hemolysis time, $t_{1/2}$ in minutes), thus, reflects the overall antioxidant potential KRL

The results between the group of drinking subjects versus the group of abstinent subjects showed an increase in the HDL level, a decrease in the LDL level, an increase in the antioxidant potential, an increase in the fluidity of the erythrocyte membrane. The results in the group of drinking subjects between D0 and D14 showed a decrease in cholesterol and LDL, an increase in the antioxidant potential

of the serum, an increase in the fluidity of the erythrocyte membrane, and an increase in the VO_2 max (maximum oxygen consumption) of 26%. A decrease in LDL and an increase in antioxidant serum are two protective factors. If there is less LDL and they are less oxidized, they will be deposited less in the arteries, so the formation of atheroma plaque will be delayed. It is possible to hope that the significant antioxidant effect obtained as early as D15, as well as the decrease in LDL levels, will have a retarding effect on the progression of the atheroma plaque. Similarly, the increase in membrane fluidity allows red blood cells to circulate better through an atheromatous artery.

3.1. Self-Action of Ethanol

High Density Lipoprotein rate increase: The oldest known mechanism is the increase in high density lipoprotein (HDL) levels [45–47], including the HDL2 and HDL3 fractions, as well as the protein constituents of HDL, Apo A1, and Apo A2 [48]. A decrease in the activity of the cholesterol transfer protein esterified, CETP [49] could be the origin of the rise of HDL. Prospective studies have found an inverse correlation between HDL levels or its fractions and the risk of ischemic heart disease [50]. Indeed, HDL is a protective factor against the formation of atheroma due to a transport of cholesterol from the vascular walls into the liver, with a biliary elimination. An increase in the HDL-linked enzyme paraoxonase protects against the LDL oxidation [51], without this increase depending on the type of alcoholic beverage. On the other hand, at high doses, ethanol increases the triglyceride level and remains neutral on the LDL level.

A decrease in platelet aggregation: Platelet aggregation is decreased by low or moderate doses of alcohol [52,53]. However, after heavy ingestion of alcohol, a rebound effect on the platelet response can be observed [54], causing sudden death.

A decrease in fibrinogen: An increase in blood fibrinogen is considered a risk factor for ischemic cardiovascular disease. According to Ridker's study [55], the concentration of fibrinogen correlates positively with alcohol consumption, with a U-shaped curve (higher concentrations for non-drinkers and those who drink more than 60 g/day alcohol). This association was valid for wine and spirits, but not for beer and cider. A low level of fibrinogen in moderate-drinkers may explain the reduction of ischemic heart diseases in the latter.

Thrombolysis: Stimulation of plasminogen activator has been observed [56].

Tissue factor: A reduction of this protein, which probably plays an important role in atherogenesis, has been reported in regular drinkers [57].

Homocysteinemia: Plasma elevation of this amino acid is considered a risk factor for cardiovascular disease. Some studies have shown a decrease in plasma levels of homocysteine in very low or moderate alcohol users [58]. However, several studies show an increase in plasma homocysteine, occurring during the consumption of alcoholic beverages, regardless of the amount of alcohol consumed [59], which goes against the previous results.

Effects on blood pressure: Substantial alcohol consumption is accompanied by a rise in blood pressure [60] but for the doses we are interested in here, the effect is rather opposite, through arterial vasodilatation.

3.2. Self-Action of Polyphenols

Phenolic compounds are classified into cinnamic acids, benzoic acids, stilbenes, lignans and flavonoids (themselves classified as anthocyanidins, flavanols, flavones, flavonols, and isoflavones) [61]. Epidemiological studies have correlated the consumption of plant polyphenols with a low incidence of coronary heart disease. The polyphenols in red wine have antioxidant and free radical scavenging properties. They protect LDL against oxidation. These polyphenols decrease platelet aggregation and inhibit the proliferation of vascular smooth muscle cells. Polyphenols stimulate the production of relaxing factors of vascular endothelium, such as nitric oxide. Finally, they contribute to preserving the integrity of the vascular endothelium by acting on both proliferation, migration, and apoptosis of endothelial cells. All these properties confer on the polyphenols the possibility of interfering with the

atherogenic process or the thrombotic phenomena associated with atherosclerosis, and could explain the vasculo- and cardio-protective effects of these compounds [62] Since wine-induced cardiovascular protection appeared to be superior to that of other alcoholic beverages, many authors have sought to demonstrate that this was a consequence of the wine's high antioxidant content [63–65]. Indeed, the red wine contains seven to ten times more tannins than the white wine, and according to the grape varieties there are different levels of polyphenols. Resveratrol and flavonoids are the two most-studied molecules today.

Reduction of LDL oxidation: In vitro, the fact that antioxidants (vitamin E, carotenoids, polyphenols, vitamin C, etc.) slow the oxidation of LDL induced by Cu^{2+} when they have been absorbed at high doses by humans, has been demonstrated many times [66,67]. Fuhrman [68] showed that subjects consuming red wine had LDL that was more resistant to oxidation. Kanner's team [69] shows that during a high-fat meal (red meat and fries), the absorption rate of malondialdehyde, an oxidative substance produced during triglyceride degradation and responsible for peroxidation LDL in the blood, was reduced by 75%, if there is consumption of red wine (or green tea), during the meal! This rate goes to 0% absorption if the meat was marinated in wine, before cooking. We have here an attractive explanation of the French paradox. These researchers describe the stomach as a bio-reactor in which the wine prevents the oxidation of fats. This study confirms the recommendation of drinking during meals. Blache, shows that oxidized HDL lose much of their property in removing cellular cholesterol [70]. He concludes that HDL, like LDL, are sensitive to oxidation and that antioxidants can provide protection by preserving their functional abilities. Resveratrol lowers the triglyceride levels [71].

Action on platelet aggregation: This was Renaud's initial hypothesis [72] to explain the French paradox. Resveratrol is also capable of inhibiting platelet aggregation [73] by inhibiting the production of cyclooxygenase, resulting in a decreased thromboxane formation [74,75]. The effects are close to those obtained with aspirin. In a study conducted at the University of Burgundy, it was shown that a wine rich in resveratrol was more effective on the parameters of atherothrombogenesis than a wine poor in resveratrol, in volunteers who ingested three glasses per day, during two weeks. Similar results have been demonstrated in vitro, with flavonoids such as quercetin [76].

Atherogenesis: The antioxidant action of polyphenols appears to be targeted on LDL, as recalled by the classically accepted physiopathology of atheromatous plaque. If paradoxically, the oxygen we breathe has a vital role for our survival, its oxygenated derivatives, free radicals, are the cause of the multiple phenomena that underlie cellular aging in general, pathologies and atherogenesis, in particular. Many factors and cells intervene in this mechanism, not all of them are clear. The vasodilator effect of NO, whose secretion is mediated by polyphenols via the NO-syntase, is one of them [76]. New data on the mode of action of polyphenols is challenging the paradigm of antioxidant flavonoids and vasodilator alcohol. Indeed, the bioavailability of these molecules is so low that their mode of action as a direct antioxidant scavenger, is unlikely, in the blood [42].

4. Polyphenols and Cancer

Polyphenols intervene, partly, by action on the estrogen receptors. For example, tea polyphenols have actions on the different stages of cancer development by blocking initiation, promotion, and progression [77]. Many studies show a modulatory effect of flavonoids on the mechanisms of apoptosis [78]. Chalopin, in 2010, discovered that polyphenols could act via the estrogen receptors [79,80]. This explains the relative protection of women, during periods of genital activity. If the process by which the isoflavones of foods interact with breast cancer cells is unclear, the research points to antioxidant, anti-inflammatory, anti-angiogenic effects that, therefore, influence the survival and growth of the tumor. It is a demonstration of the influence of certain lifestyle factors, including nutrition, on cancer survival, after diagnosis. Survival is better in patients who consume more natural dietary isoflavones (and not isoflavone supplements) [81]. Flavonoids also exert an anti-aromatase action and may reduce the proliferative effect of estrogens, in this way, in the case

of hormone-dependent cancers [82]. While the protective effect is fully demonstrated for Asian populations consuming soy isoflavones since childhood, the effect on European populations has not been demonstrated, but there is a possible evidence to support efficiency. In fact, moderate consumption of wine seems to be beneficial for both men and women. One of action seems to be via the estrogen receptors, which could explain the positive effect outside of hormone-dependent breast cancer [83]. A particular point on resveratrol, this stilbene, which has been the most studied. Its anti-cancer effect in vitro has been studied by many teams, with a protective action in the three stages of carcinogenesis—initiation, promotion, and progression [84]. Resveratrol has a multi-organ action, it acts on inflammation and free radicals, it is immunosuppressive, antitumoral, and its actions go through signaling pathways, estrogen receptors, and sirtuins [85].

5. Polyphenols and Metabolism

Foxo1 is a transcription factor of the Forkhead box family (FOXO) that regulates various signaling pathways, including oxidative stress, programmed cell death, catabolism, and insulin sensitivity [86]. The transcription factor Foxo1 is a key player in insulin transcriptional responses and plays a central role in metabolic adaptation during fasting. The major role of Foxo1 and its counterparts is protection against oxidative stress and DNA damage, and thus in determining longevity. The binding of insulin to its receptor triggers a series of phosphorylation—in the order IRS, PI3K, Akt, and Foxo1. Phosphorylated Foxo1 can no longer migrate to the nucleus to induce transcriptional activation. The Sir2 (silencing information regulator) gene is capable of increasing the number of divisions of the same cell by, approximately, 30%. Mammals have 7 Sir2 homologs called sirtuins (SIRT1-7), and it is the SIRT1 protein that appears to be functionally closest to sir2. SIRT1 "silent information regulator 1", is a NAD-dependent deacetylase, whose activity, therefore, directly depends on the nutritional state since it varies the NAD/NADH ratio. The longevity caused by caloric restriction, a condition that increases the NAD/NADH ratio, would at least, partly, pass through Sir2/SIRT1. It is involved in various processes [87,88]—inflammation, energy restriction, mitochondria biogenesis, stress resistance, cellular senescence, endothelial function, apoptosis, and circadian rhythm. It decreases transcription of the P53 protein, which decreases apoptosis [89]. The E2F1 protein stimulates the expression of sirtuin 1, which in turn inhibits the activity of the first and its apoptotic activity. It is also, by this means, protective against DNA damage [90]. By combining with a FOXO protein, it protects the cell against oxidative stress [91]. In mice, with a mutation in the SIRT1 gene resulting in the production of a non-functional protein, lethality is important in utero, with surviving individuals showing multiple tumors, probably secondary to an inability to repair DNA. [92]. SIRT1 can, therefore, be considered a tumor suppressor gene.

The effect of resveratrol on sirtuins would produce an effect mimicking caloric restriction and increasing longevity, a beneficial effect on type 2 diabetes also seems possible, by increasing the insulin sensitivity and lowering the blood glucose [93,94]. Low wine consumption would increase life expectancy by five years [95]. It has long been shown that caloric restriction is in itself one of the criteria for extending life expectancy, universally [96,97]. The principle of caloric restriction is to leave the table without having access to the sensation of satiety or by accessing it by a high-consumption of foods with a low-caloric index.

The Cretan diet is the combination of a frugal diet, low calories (caloric restriction), and the Mediterranean diet type. Explaining the excellent life expectancy of the Cretans.

Fifteen percent of the world's super-centenarians (over 107 years old) live on the island of Okinawa. The Okinawa diet is the application, with a Japanese diet, of an organized calorie restriction:

- Stop eating before being completely satiated (Hara Hachi Bu).
- Eat only small portions (kuten gwa).
- Eat with the thought that food has healing powers (nuchi gusui).
- Promote a variety of foods.

- Eat fresh foods.
- Combine raw and cooked foods.
- Cook little food over low heat.
- Avoid the microwave oven and barbecue.
- Give preference to colors on the plate.

This action of polyphenols on sirtuins is the key to protecting against diabetes and increasing the longevity of moderate wine drinkers.

6. Polyphenols and Alzheimer

Alzheimer's disease is an incurable neurodegenerative disease of brain tissue that results in the progressive and irreversible loss of mental functions, including memory. It is the most common cause of dementia in humans. It was initially described by the German doctor Alois Alzheimer in 1906. Two types of nerve tissue damage characterize Alzheimer's disease: Senile plaques (or amyloid deposits) and neurofibrillary degeneration. The constituents of these lesions are, respectively, the amyloid peptide (or Aβ) and the Tau protein. The positive effect of wine on neurodegeneration has been confirmed by numerous studies, confirming that alcohol has a more general vascular action. The PAQUID (Personnes Agees QUID) study [98,99] identified the main neuroprotective factors, of which wine is a part. Here again, we fear an association bias between wine consumption and socio-economic levels, with favorable dietary rules. Over thirteen years of study, in patients aged over 65 years in the Gironde and Dordogne, classified as non-drinkers, light-drinkers, moderate-drinkers and heavy-drinkers, it appeared that there was no difference between the non-drinkers and the light-drinkers, for risk of Alzheimer's disease. It is from three or four glasses a day that the benefit appears and increases with time. This study showed a lower decline in cognitive function in subjects drinking moderately, while it was generally agreed that even at very low doses, alcohol consumption could induce cognitive impairment. A study conducted in Rotterdam [100], on 5400 subjects over 55 years, concluded that vascular dementia is also less common among drinkers, whether beer or wine. A study also confirms this in women [51,101].

Moderate alcohol consumption improves the mood and quality of life of older men and women [102], by promoting better sociability. It also helps stimulate appetite. Resveratrol decreases the secretion of beta amyloid protein in rats [103]. The Bordeaux study of Orgogozo and Dartigues [99] showed a significant decrease in dementia and Alzheimer's disease for consumption of 4 to 5 glasses of red wine, per day.

7. Hormesis

Hormesis designates a response of stimulation of the biological defenses, generally favorable, with exposures of low doses of toxins or other agents or phenomena stress generators. As a result of this mechanism, some natural toxins or pollutants may have the opposite effect, depending on whether the dose received is low or high. These agents are said to be hormetic. In toxicology, the phenomenon of hormesis is characterized by a characteristic form of the dose/effect relationship curve, which changes sign for low doses, giving it a "U" or "J" shape (Figure 9).

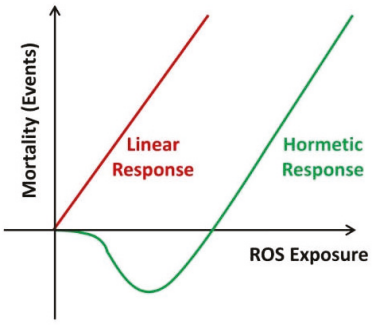

Figure 9. Hormetic response.

It is usually accepted as a dose-dependent response to a stimulus. The current hypothesis is rather a response to a prooxidant background mediated by nrf2 (nuclear respiratory factor 2) [103]. Normally, moderate oxidative stress will favor the nrf2 pathway responsible for the endogenous synthesis of anti-oxidant enzymes. In case of overflow, it is the inflammatory NFkb pathway that will be triggered with a passage to apoptosis [104].

It is interesting to note that the famous curve in U (or in J), corresponds to a hormetic profile. It is, therefore, in small doses that alcohol and wine must be consumed, in order to have a beneficial effect on health [105,106] (Figure 10).

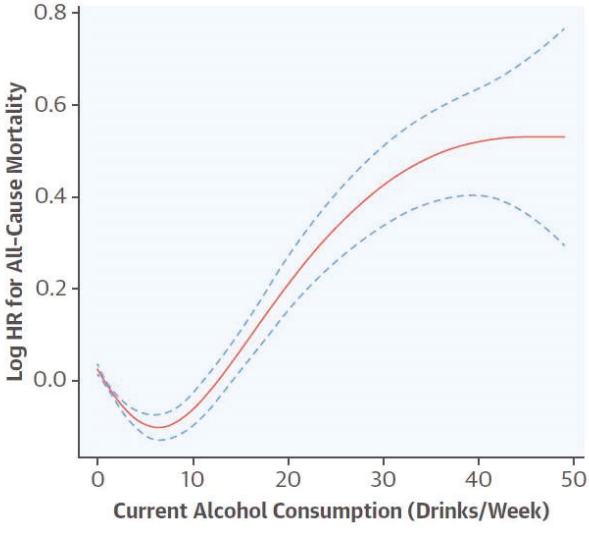

Figure 10. The new J curve [107].

Bo showed, in 2017, that the association between alcohol consumption and mortality risk in U.S. adults, follow a J-shaped curve. Compared to abstainers, the all-risk mortality of moderate consumers, were reduced. For heavy alcohol consumption the risk increased significantly [107].

Therefore, there are good ways to hope to understand the action of wine polyphenols on human health. First, there is a direct antioxidant effect (scavenger) when wine is absorbed during the meal, after ingestion, polyphenols seems to act as prooxidant. This prooxidant impregnation mechanism, involving the epigenetic expression of endogenous antioxidant enzymes.

8. Conclusions

Wine has always been part of the culture of human being, from the east where it was born, to the west where it has taken all its colors and flavors. As far as we can read its footsteps, we can measure its place, both, in the sharing between men and in the benevolent health that fall in the scriptures. Today all scientific studies demonstrate the undeniable part of the quality of nutrition in human health. Wine is unquestionably a health food, on the condition that it is consumed, or is rather savored, in moderation and as a point of conviviality. Current data make it an antioxidant, a repairer of cell damage, a cardiovascular protector, a metabolic and neurological protector [108,109]. Especially wine, in a moderate dose, brings the pleasure of an exchange between guests around a good meal. It is no coincidence that the gastronomic meal of the French was included in the UNESCO's universal heritage in 2010.

After the study published in the Lancet [110], all alcoholics shouted victory. We are told that wine is an alcohol like the others, and especially that we will die from the first glass of alcohol.

This reminds us of a leaflet from the INCA (The French National Institute of Cancer) in 2009, which has already promised us cancer from the first glass of wine. However, at the same time, Lanzmann-Petithory said, "men who consume mostly wine have a risk of premature mortality from all causes decreased by 25%" [111].

Fortunately, other researchers, but those we talk a little less of, prove other truths. Over ninety studies conducted since 1981 by Dr. Claudia Kawas, 14,000 Californian retirees were followed, the results, as early as 2007, show that men and women who drink alcohol have a lower risk of mortality than the abstainers (those who drank one or two glasses a day saw their mortality reduced by 15%). The study continues and in February 2018, at the American Association for the Advancement of Science conference in Austin, Texas, the results were confirmed—the consumption of two glasses of alcohol per day is associated with a reduction of 18% of the risk of death, compared to abstainers [112]. The study also confirmed that regular exercise, social activities, the regular practice of a hobby and coffee consumption, also increase the life span.

As already mentioned Alvise Cornaro, a Venetian who, after having abused the pleasures of the flesh for 40 years, was given lost by medicine, he was finally able to live to 103 years, after questioning his way of life (frugal food and a little bit of wine). He said: "What we leave after a hearty meal does us more good than we ate" [113].

Wine is the best of anxiolytics. Allowing citizens to relax with a glass of wine, rather than making them feel guilty, could reduce prescriptions for benzodiazepines and antidepressants; while eliminating the side effects of these dangerous drugs. Wine is our panacea. Only misuse or abuse, is dangerous. In conclusion, let us drink fewer, but drink better to live older.

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Conflicts of Interest: The author declares no conflict of interest.

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Review

Wine: An Aspiring Agent in Promoting Longevity and Preventing Chronic Diseases

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Abstract: Introduction: Moderate wine consumption is a characteristic of the Mediterranean diet. Studies around the world have shown a beneficial effect of moderate alcohol intake, especially wine, on health. This review aims to critically summarise the most recent studies that investigate the beneficial effects of moderate wine intake on human health. Methods: The PubMed database was comprehensively searched to identify trials published from 2013 to 2018 that investigated the association between moderate wine consumption and health. Results: The most recent studies confirm the valuable role of moderate wine consumption, especially red wine, in the prevention and treatment of chronic diseases such as cardiovascular disease, metabolic syndrome, cognitive decline, depression, and cancer. In the meantime, recent studies also highlight the beneficial role of red wine against oxidative stress and in favour of desirable gut bacteria. The beneficial role of red wine has been attributed to its phytochemical compounds, as highlighted by clinical trials, where the effect of red wine has been compared to white wine, non-alcoholic wine, other alcoholic drinks, and water. Conclusions: Moderate wine intake, at 1–2 glasses per day as part of the Mediterranean diet, has been positively associated with human health promotion, disease prevention, and disease prognosis.

Keywords: wine; vine; diet; health; dementia; cardiovascular disease

1. Introduction

Moderate wine consumption is a characteristic of the Mediterranean diet, and was first described by Ancel Keys in the Seven Countries study. High adherence to this dietary model is associated with reduced risk for various chronic diseases [1–5]. Moderate alcohol consumption, especially wine [6], is generally regarded to be beneficial to health [7,8]. Other alcoholic drinks, such as beer, also seem to improve the lipid profile and other factors related to atherosclerosis [9].

In fact, the "French paradox" [10] suggests that red wine consumption is the reason for lower ischaemic heart disease mortality in the French population, where high saturated fat consumption is observed [10], although some scientists support that the benefit should be attributed to alcoholic drinks in general [11].

Despite certain health benefits, alcohol consumption has been considered detrimental for public health in general [12]. Additionally, binge drinking [7] and high alcohol intake [13] have also been associated with negative health impacts. In fact, 88,000 deaths in the United States each year are attributed to excessive alcohol intake, representing 1 in 10 deaths among adults aged 20–64 years old [14]. Although moderate alcohol consumption reduces cardiovascular mortality, individual

characteristics and dietary habits should be taken into account, as decreases in alcohol consumption are related to general health benefits [13].

Alcohol consumption guidelines vary between countries [11]. In the USA, alcohol consumption is recommended not to exceed one drink per day for women and two drinks per day for men, where one drink is described to contain 14 g of pure alcohol [15]. In the United Kingdom, it is recommended that both men and women drink no more than 14 units alcohol per week, where one unit is equivalent to 10 mL of pure alcohol [16]. Guidelines highlight that people who do not drink alcohol should not be advised to start drinking, while alcohol consumption should be part of a healthy eating pattern [17].

Over the last decades, disease patterns have changed, with non-communicable diseases being on the increase and the communicable diseases on the decrease. Current diseases, such as cardiovascular diseases, cognitive impairment, and cancer, and their prevention have been associated with environmental factors such as diet and lifestyle. Alcohol and wine have been studied over the years with respect to the health benefits and risks their consumption confers.

Several epidemiological and clinical studies have attributed wine (specifically its phytochemical components such as resveratrol, quercetin, polyphenols, and flavonoids) as having positive effects on health, health promotion, disease prevention, and disease prognosis [8,11].

However, the 15-year experience gained from the Seven Countries Study, which enrolled 11,579 healthy men aged 40–59 years old and recorded 2,289 deaths due to coronary heart disease (CHD), suggests the significance of the investigation of many other factors for safe conclusions. Factors such as age, smoking, serum cholesterol, blood pressure, body mass index (BMI), and physical activity play a very important role. There are also some other important factors, such as socioeconomic status or ethnic differences, which are often not taken into account and can affect diet and other aspects of life [18]. Thus, findings should be taken cautiously.

The present study aims to critically collect and summarise in depth the most recent clinical data to highlight the association between wine consumption and health.

2. Methods

Observational studies and clinical trials investigating the relation between wine consumption and health were thoroughly searched in the PubMed database using relative key words for studies published from 2013 to April 2018. The key words were, "wine health benefits", "wine" and "cardiovascular disease", "blood pressure", "metabolic syndrome", "weight", "cancer", and "mental health". Only studies conducted in humans were taken into account. The primary search resulted in more than 120 publications, which were then evaluated. The secondary search was restricted to clinical trials, reviews, and meta-analyses written in English with titles including wines and selected diseases. Finally, 54 studies fulfilling the criteria were selected. Studies that were focused on the influence of alcohol on the human body and those focused only on the biochemical ingredients of grapes were excluded. Thus, 65 other studies, referring to the impact of moderate wine consumption, were taken into account and summarized.

3. Results

3.1. Association between Wine Consumption and Cardiovascular Disease

In the last five years, 10 studies have investigated the role of wine consumption on cardiovascular disease (Table 1).

Table 1. Association between wine consumption and cardiovascular disease [17–26].

Clinical Sample	Dose	Duration-Experimental Models	Main Results	References
1248 patients	Wine $\leq$ 500 mL/day	3.5 years of follow-up	Lower risk of cardiovascular (CV) events and mortality	Levantesi G., et al., 2013
6973 patients	1 glass of wine per day	Kansas City Cardiomyopathy Questionnaire	Better health status, lower depressive symptoms and vascular inflammation	Cosmi F., et al., 2015
449 older, U.S. male physicians with prevalent heart failure	1–2 drinks per day (beer, wine, or liquor)	7 years	Lowest mortality, independent of alcoholic drink type	Petrone A.B., et al., 2014
11,470 patients with type 2 diabetes aged at least 55 years in 20 countries	0.28 L of beer, 125 mL of wine, and 25 mL of spirits.	5 years of follow-up, self-report	Reduced risks of CV events and all-cause mortality	Blomster J.I., et al., 2014
40 otherwise healthy individuals with high cholesterol	RW, 125 mL for women and 250 mL for men/daily	1 month	Better cholesterol levels and LDL/HDL ratio	Apostolidou C., et al., 2015
23 hypercholesteraemic participants	250 mL daily of RW or WW or RO	10 weeks	Both RW and RO improved LDL oxidation lag time.	Chiu H.F., et al., 2016
12 healthy men, aged 25–39 years	4 mL/kg body weight WW or RW or ethanol solution	2 weeks	Cardioprotective effect of moderate wine consumption, independently of ethanol.	Xanthopoulou M.N., et al., 2017
157 healthy participants	RW and WW	12 months	Both RW and RO improve LDL. RW suppressed the total cholesterol	Taborsky M., et al., 2017
122 patients, aged >30 years	RW, women: 100 mL, men: 200 mL, with rich Med.	20 weeks	No changes on peak systolic, end-diastolic or mean cerebral blood flow velocity	Droste D.W., et al., 2014
122 patients, aged >30 years	RW (100 mL for women and 200 mL for men)	20 weeks	Improved the LDL/HDL ratio of the participants	Droste D.W., et al., 2013

RW, red wine; WW, white wine; RO, extract of onion; CV, cardiovascular; CHOL, cholesterol; Med., Mediterranean Diet.

Levantesi et al. [19] aimed to investigate the associations of wine intake, cardiovascular events and total mortality in 1,248 patients after myocardial infarction, in subjects enrolled in the Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardico (GISSI)-Prevenzione Trial. Moderate wine consumption at $\leq$500 mL/day was associated with lower risk of cardiovascular events and mortality, as compared to not drinking wine after 3.5 years of follow-up [19].

In 6973 patients with chronic heart failure enrolled in the GISSI-Heart Failure (GISSI-HF) trial, moderate wine consumption of one glass per day was associated with a better health status according to the Kansas City Cardiomyopathy Questionnaire, lower depressive symptoms according to the Geriatric Depression Scale, and lower vascular inflammation, yet without improved 4-year clinical outcomes [20]. However, in 449 older (75.7 $\pm$ 8.2 years old) U.S. male physicians with prevalent heart failure who were followed for 7 years, a J-shaped association between alcohol consumption and mortality was observed, with the lowest mortality at 1–2 drinks per day independent of alcoholic drink type (beer, wine, or liquor) [21].

In patients with type 2 diabetes, the association between alcohol consumption and cardiovascular disease is not clear. Blomster et al. [22] undertook a study to investigate the role of moderate alcohol consumption on cardiovascular health. After 5 years of follow-up, compared to those who did not drink alcohol, those who had moderate alcohol consumption had fewer cardiovascular events and microvascular complications, and lower all-cause mortality. Those who mainly drunk wine benefited the most [22].

Apostolidou et al. [23] studied the effects of moderate wine consumption in 40 otherwise healthy individuals with high cholesterol. In this crossover study participants consumed either "tannat" red wine or a placebo drink (125 mL for women and 250 mL for men daily) for one month. The antioxidant capacity and vitamin E levels of both patients with high cholesterol and subjects with normal cholesterol were improved, while in patients with high cholesterol the fasting LDL/HDL ratio was also improved [23]. Chiu et al. [24] investigated the effect of red wine extract of onion and red wine on cardiovascular disease risk factors. Twenty-three hypercholesteraemic participants were randomised to consume 250 mL daily of red wine or red wine extract of onion for 10 weeks. As a result, the body's antioxidant capacity was increased, delaying the oxidation of LDL cholesterol. However, compared to red wine, the red wine onion extract had an additional hypocholesterolaemic effect, lowering total and LDL cholesterol while modulating inflammation and factor VII, and showing a better cardioprotective effect [24].

The role of wine consumption on platelet aggregation against platelet-activating factor was investigated in a small cross-over study with 12 healthy men [25]. Participants consumed a standardised meal along with either white wine, red wine, ethanol, or water. A significant effect was found in platelet sensitivity against the platelet-activating factor, with greater effect after red wine consumption, compared to both ethanol and water. The plasminogen activator inhibitor-1 concentration was higher for all alcoholic beverages compared to water, while triglycerides were only increased significantly after ethanol consumption as compared to water. Red and white wine lowered postprandial triglyceride concentrations [25]. In the Vino Veritas randomised trial the effects of red and white wine on atherosclerosis were investigated [26]. In this study, 157 healthy participants were randomised to receive either white or red wine for one year. After 12 months, LDL was similarly decreased in both groups, while total cholesterol was lowered in the red wine group, but the levels did not differ from the white wine group. In general this study failed to show positive clinical differences in atherosclerosis markers in healthy participants [26].

In patients with carotid atherosclerosis, the combination of a polyphenol-rich Mediterranean diet and moderate daily physical activity (30 min/day) with moderate red wine consumption (100 mL for women and 200 mL for men) for 20 weeks did not affect the middle cerebral and internal carotid blood flow velocity [27]. The absence of significant improvement may be due to the fact that 66% of the study population was on statin therapy [27]. However, in the same patient population, these lifestyle

changes (healthy diet plus red wine, and exercise) for 20 weeks did improve the LDL/HDL ratio of the participants [28].

The review by Fernández-Solà [13] refers to epidemiological studies, case–control studies, and meta-analyses that indicate a U-type dose-dependent bimodal relationship between alcohol consumption (mainly wine and beer) and cardiovascular health. On the basis of these analyses, the reductions of cardiovascular events and mortality are associated only with low to moderate alcohol consumption, as compared to abstinence. This relationship was also reported in the 4th century BC, by Hippocrates, to emphasize the harmful effects of alcohol abuse on the heart. It is further argued that initiation of alcohol consumption for the benefit of health should not be encouraged, as a substantial number of diseases are associated with the harmful effects of high alcohol consumption. The beneficial and negative effects of alcohol consumption, as well as the overall lifestyle of the individual (smoking, lack of exercise, eating habits, etc.) should always be taken into account and evaluated [13].

3.2. Association between Wine Consumption and Blood Pressure

Seven interventional studies have been undertaken since 2013 to study the effect of wine consumption and blood pressure (Table 2).

Table 2. Association between wine consumption and blood pressure [27–33].

Clinical Sample	Dose	Duration-Experimental Models	Main Results	References
24 premenopausal women, aged 25–49 years	200 to 300 mL red wine (RW)/day	4 weeks	Increased the 24-h systolic and diastolic BP.	Mori T.A., et al., 2015
24 patients with well-controlled T2DM	Women: 230 mL RW/day, Men: 300 mL/day or DRW	4 weeks	RW, increased heart rate (HR), awake and asleep (24 h)	Mori T.A., et al., 2016
54 participants (age = 57 years; 85% men) with T2DM	150 mL RW at dinner/daily, with Med. diet	6 months	Reductions in BP were observed in the red wine group at midnight (3–4 h after ingestion)	Gepner Y., et al., 2016
224 patients with T2DM	150 mL of mineral water, WW, or RW with dinner	2 years	No differences were identified in blood pressure	Gepner Y., et al., 2015
18 healthy subjects, aged 25–53 years)	2 glasses of RW	24 h	Higher heart rate during the consumption and lowered after consumption	Fantin F., et al., 2016
25 normotensive men, aged 20–65 years	375 mL RW or 375 mL non-alcoholic wine or water	3 different days	A decrease in BP in the first 4 h and an increase after 20 h	Barden A.E., et al., 2013
60 untreated, hypertensive participants	2 grape extracts (grape-RW and grape alone)	4 weeks	Systolic and diastolic BP were significantly lower during the day.	Draijer R., et al., 2015

BP, blood pressure; RW, red wine; WW, white wine; DRW, dealcoholized RW; T2DM, type 2 diabetes.

In healthy women the dose-dependent association between wine consumption and blood pressure was investigated by Mori et al. [29]. Healthy premenopausal women were randomised to receive red wine in higher or lower levels than usual, or dealcoholized red wine for 4 weeks. When consuming higher volumes of wine than usual both diastolic and systolic blood pressure were elevated. This study showed that, like in men, 200–300 mL red wine per day was found to increase the 24-h diastolic and systolic blood pressure, as compared to dealcoholized wine [29].

Mori et al. [30] undertook another small randomised three-period crossover study in 24 patients with well-controlled type 2 diabetes [30], and examined the role of wine consumption on cardiovascular risk factors. Women were randomised to drink 230 mL/day red wine and men 300 mL/day red wine,

or equivalent volumes of dealcoholized red wine or water, for 4 weeks. Red wine increased awake systolic and diastolic blood pressure, as compared to water. Diastolic blood pressure during sleep was decreased after red wine compared to dealcoholized red wine. Red wine increased the heart rate while subjects were sleeping, awake, and overall over 24 h, as compared to water and dealcoholized red wine. Compared to dealcoholized red wine, red wine did not affect glycaemic control or cardiovascular risk factors [30]. Gepner et al. [31] in their study had different results. In 54 participants (age = 57 years; 85% men) with type 2 diabetes who did not drink alcohol, a daily consumption of 150 mL red wine at dinner combined with a Mediterranean diet for 6 months did not change the median 24-h blood pressure, but did lower the blood pressure 3–4 h after wine consumption, at midnight (3–4 h after ingestion). In subjects homozygous for the gene encoding the ADH1B*2 variant that leads to fast alcohol metabolism, the median 24-h blood pressure and pulse pressure values were decreased compared to heterozygotes and those homozygous for the ADH1B*1 variant (slow alcohol metabolisers) [31]. In another 2-year-long study by Gepner et al. [32] in alcohol-abstaining patients with well-controlled type 2 diabetes, blood pressure remained unchanged after wine consumption initiation at 150 mL per day along with dinner [32].

In healthy subjects, two glasses of wine lead to a higher heart rate during the consumption, and lowered arterial compliance after consumption [33]. Eighteen healthy volunteers received one drink with alcohol (two glasses of red wine) and one drink without alcohol on two consecutive, but separate days. Red wine increased heart rate during alcohol ingestion, and reduced arterial compliance after ingestion. The day and ingestion period were found to have significant effects on heart rate, diastolic blood pressure and QKD, suggesting that the differences in response among the ingestion periods depended on whether alcohol had been consumed that day. For the first time their study indicates the effect of alcohol on 24-h arterial stiffness in a healthy group of volunteers [33].

Although chronic wine consumption and hypertension have been correlated, vasodilation after wine ingestion has also been observed. In a study by Barden et al. [34] consumption of 375 mL red wine (41 g alcohol) or 375 mL non-alcoholic wine or water along with light dinner on three different days was investigated in order to assess the effects of wine on blood pressure. Red wine consumption led to a decrease of blood pressure the first 4 h and then an increase after 20 h. The vasoconstrictor 20-hydroxyeicosatrienoic acid (20-HETE) was reduced 2 h after the ingestion of either drink, but was increased within 24-h after red wine consumption. The time point for the lowest levels of 20-HETE, at 2 h after red wine ingestion, is the time point when the highest alcohol levels in the blood are measured, indicating an important homeostatic response [34].

Draijer et al. [35] undertook a double-blind placebo controlled crossover study in order to assess the effect of two grape extracts (grape/red wine and grape alone) on blood pressure and vascular function in 60 untreated, mildly hypertensive participants for 4 weeks. Both extracts had high concentrations of anthocyanins and flavonols, but the control drink (grape alone) was relatively poor in catechins and procyanidins. The 24-h ambulatory systolic and diastolic blood pressures were significantly lower in the grape-wine extract intervention as compared to placebo, predominantly during the day. Plasma concentrations of the vasoconstrictor endothelin-1 decreased by 10%, but other measures of vascular function were not affected. The control drink had no effect on blood pressure and on vascular function, indicating an important role for catechins and procyanidins on blood pressure [35].

3.3. Association between Wine Consumption and Metabolic Syndrome

With respect to metabolic syndrome (MS), eight studies investigated the impact of wine on MS and its constituents (Table 3).

Table 3. Association between wine consumption and metabolic syndrome (MetS) [34–41].

Clinical Sample	Dose	Duration-Experimental Models	Main Results	References
15,905 Hispanics/Latinos, aged 18–74 years	RW, WW, beer, liquor	Self-report questionnaire	Low levels of wine, related with lower odds of MetS	Vidot D.C., et al., 2016
64,046 participants aged 18–80 years	Beer, wine or spirits/mixed drinks group	Self-report questionnaire	Protective effect against MetS, and low HDL cholesterol	Slagter S.N., et al., 2014
14,375 active or retired civil servants, aged 35–74 years	Beer (350 mL), wine (120–150 mL) or spirits (40 mL).	Standard questionnaire	Consumption of wine in lesser quantities with meals was generally more protective than when taken outside of meals	Vieira B.A., et al., 2016
8103 participants (men = 2687 and women = 5416)	Red or other wines (100 mL), beer (330 mL), and spirits (50 mL)	Questionnaire consumption	Higher risk of developing specific MetS after at least 6 years of follow-up with 7 alcoholic drinks/week	Barrio-Lopez M.T., et al., 2013
5801 elderly participants at a high cardiovascular risk	100 mL of wine, 250 mL of beer, 65 mL of liquors and 32 mL of spirits	137-item FF Questionnaire	Lower prevalence of the MetS in an elderly Mediterranean population at a high cardiovascular risk	Tresserra-Rimbau A., et al., 2015
66,485 women from the French prospective E3N-EPIC cohort	150 mL wine, 250 mL beer, 70 mL fortified wine, 40 mL spirits	Questionnaires, every 2–3 years, for 14 years	Wine, associated with T2D risk, only in overweight women.	Fagherazzi G., et al., 2014
67 men at high cardiovascular risk, after a run-in period	RW (30 g alcohol/day) or gin (30 g alcohol/day)	4 weeks	Beneficial effect of the non-alcoholic fraction of RW on insulin resistance and cardiovascular disease	Chiva-Blanch G., et al., 2013
224 patients with T2DM	150 mL of mineral water, WW, or RW with dinner	2 years	Both ethanol and RW non-alcoholic constituents can be beneficial to the cardio-metabolic risk in well controlled T2D patients.	Gepner Y., et al., 2015

FFQ, Food Frequency Questionnaire; MetS, metabolic syndrome; RW, red wine; WW, white wine; DRW, dealcoholized RW; T2D, type 2 diabetes. E3N, Etude Epidémiologique auprès de femmes de la Mutuelle Générale de l'Education Nationale; EPIC, European Prospective Investigation into Cancer and Nutrition.

The prospective, population-based, cohort study Hispanic Community Health Study/Study of Latinos (HCHS/SOL), with data from 15,905 participants showed that low and moderate wine consumption was independently associated with lower risk of metabolic syndrome than alcohol abstinence [36]. The LifeLines cohort study also indicated a protective effect of wine consumption against metabolic syndrome, as well as low HDL cholesterol [37], while in the Brazilian Longitudinal Study of Adult Health (ELSA-Brasil), participants who drank mainly wine at 1–4 drinks per week were at lower risk of having metabolic syndrome [38]. On the other hand, another cohort study in younger subjects, with mean age at 35.4 years, failed to show an association between wine consumption and risk of metabolic syndrome [39].

The cross-sectional Primary Prevention of Cardiovascular Disease with a Mediterranean Diet (PREDIMED) study [40] with 5801 elderly participants at high cardiovascular risk highlighted the protective effect of red wine consumption. Compared with non-drinkers, those who drink one or more glasses red wine per day were found to have 44% lower risk of MS, 41% lower risk of high waist circumference, 58% lower HDL cholesterol, 72% lower high blood pressure, and 33% higher fasting plasma glucose after adjustment for confounders, especially in women, participants <70 years old, and those who smoked or used to smoke. Hence, moderate red wine intake can lower the risk of metabolic syndrome in elderly patients at a high cardiovascular risk. [40]. Considering type 2 diabetes, the E3N-EPIC cohort study [41] evaluated the role of wine consumption on the risk of developing diabetes in 66,485 women. In overweight women, wine consumption was negatively associated with risk of diabetes, at a consumption of two or more drinks per day, as compared to alcohol abstainers [41].

Sixty-seven men at high risk of cardiovascular disease were randomised in a crossover trial to receive red wine (30 g alcohol/day), the equivalent amount of dealcoholized red wine, and gin (30 g alcohol/day) for 4 weeks [42]. After four weeks of red wine and dealcoholized red wine consumption, although fasting glucose did not change, the mean adjusted plasma insulin and Homeostatic model assessment Insulin Resistance (HOMA-IR) were decreased, indicating a beneficial role of polyphenols on insulin sensitivity. Both red wine and gin increased HDL cholesterol, as well as apolipoprotein A-I and A-II concentrations. Lipoprotein(a) levels were decreased after the red wine consumption [42].

Well-controlled type 2 diabetes patients who did not drink alcohol were randomised to receive 150 mL of water, white or red wine with dinner for 2 years, along with Mediterranean diet. Red wine significantly increased HDL cholesterol and apolipoprotein(a)1, and decreased the total/HDL cholesterol ratio, while reducing the number of metabolic syndrome components. When ethanol metabolism genotypes were taken into account, it was observed that slow ethanol metabolisers benefited from both white and red wine in terms of glycaemic control as compared to fast ethanol metabolisers. Thus, initiation of moderate wine intake, especially red wine, can help improve the cardio-metabolic risk in well controlled diabetes patients, while with respect to glycaemic control, both ethanol and red wine's non-alcoholic constituents can be beneficial [32]. However, this trial failed to show improvements in blood pressure, adiposity, liver function, drug therapy, and symptomatology [32].

3.4. Association between Wine Consumption and Weight

Five studies investigated the role of wine intake on weight gain (Table 4).

Table 4. Association between wine consumption and weight [41–44].

Clinical Sample	Dose/Type	Duration	Main Results	References
224 patients with T2DM	150 mL of mineral water, WW, or RW with dinner	2 years	No weight change in patients with T2DM	Gepner Y., et al., 2015
48 participants	150 mL of mineral water, WW or RW with dinner	2 years	Without changes to ratio of abdominal fat	Golan R., et al., 2017
14,971 from 51,529 men in the USA, aged 40–75 years	FFQ	4 years	Dose-dependent relationship	Downer M.K., et al., 2017
5879 Australian-born individuals aged 40–69 years	121-item FFQ	8 years	Greater waist circumference and body weight	MacInnis R.J., et al., 2014
7855 men aged 50–59 years	FFQ	2 years	Associated with BMI and waist circumference	Dumesnil C., et al., 2013

FFQ, Food Frequency Questionnaire; RW, red wine; WW, white wine; DRW, dealcoholized RW; T2DM, type 2 diabetes.

Wine consumption did not affect weight change in patients with type 2 diabetes after initiating daily wine consumption for 2 years [32,43]. A cohort study in men also failed to show an association between wine consumption or increased wine consumption and weight change [44]. However, middle aged participants of the of increased body weight and waist circumference for middle-aged adults of the Melbourne Collaborative Cohort Study were less likely to have greater waist circumference and body weight when drinking low to moderate amounts of alcohol, including wine [45]. Mean BMI was lower in people who drank alcohol and wine daily than those who consumed alcohol less frequently, while for a given alcohol intake, wine (and beer) intake was inversely associated with BMI and waist circumference [46].

3.5. Association between Wine Consumption and Cancer

In the last five years, 15 studies have been published concerning the relationship between wine and cancer (Table 5).

Table 5. Association between wine consumption and cancer [45–59].

Clinical Sample	Dose/Type/Experimental Models	Main Results	References
2513 cases of ovarian cancer	Beer, RW and WW and spirits/questionnaire	Wine consumption was associated with a lower risk of cancer	Cook L.S., et al., 2016
476,160 individuals aged 35–70 years from 10 countries	Beer, wine, sweet liquor, distilled spirits/13.9 year	No association between alcohol and UCC	Botteri E., et al., 2017
301,051 women from 10 countries	Different types of alcoholic beverages/questionnaires	No associations between alcohol consumption and endometrial cancer risk.	Fedirko, V., et al., 2013
66,481 women aged 40–65 years from the French	150 mL wine, 250 mL beer, 70 mL fortified wine, 40 mL spirits/questionnaires	>2 glasses of wine/day in postmenopausal period increased the risk of breast cancer by 33%	Fagherazzi G., et al., 2015
167,765 women from the NHS and 43,697 men	Beer, RW, WW, liquor/FFQ	Alcohol consumption is associated with increased risk of cutaneous BCC	Wu S., et al., 2015
380 BCC and 390 controls with benign skin conditions	Wine, beer, hard liquor or mixed drinks/saliva sample and questionnaires	No association between lifetime alcohol intake and early-onset BCC	Zhang Y., et al., 2014.
59,575 white postmenopausal women	Beer, wine, liquor and gin, brandy and whisky/FFQ	Increased hazard of melanoma (MM) and risk of non-melanoma skin cancer	Kubo J.T., et al., 2014
210,252 participants from the USA	Beer, RW, WW, liquor/FFQ	Alcohol intake was associated with a modest increase in the risk of melanoma	Rivera A., et al., 2016
120,852 participants aged 55–69 years from Holland	Beer, RW, WW, liquor/FFQ	Wine consumption was inversely associated with overall risk of HNC, and HNC-subtypes	Maasland D.H.E., et al., 2014

Table 5. *Cont.*

Clinical Sample	Dose/Type/Experimental Models	Main Results	References
24,068 men and women aged 39–79 years	Beer, wine and spirits/FFQ	Wine drinking associated inversely with lower risk of oesophageal adenocarcinoma	Yates M., et al., 2014.
3397 patients	Beer, wine and liquor/FFQ	RW, associated with longer overall survival and disease-free survival	Phipps A.I., et al., 2016
4966 cases of incident invasive colorectal cancer (CRC)	Beer, hard cider, wine, fortified wines, hard liquor/FFQ	Wine consumption modestly associated with more favourable survival after colorectal cancer (CRC)	Phipps A.I., et al., 2017
3146 patients with CRC from southwest of Germany	Beer, wine and liquor/questionnaires	Alcohol abstinence and heavy drinking behaviour were associated with poorer survival after CRC	Walter V., et al., 2016
141 patients with incurable invasive cancer	Wine arm and nutritional supplement arm/questionnaires and diaries	Wine does not improve appetite or weight in advanced cancer patients.	Jatoi A., et al., 2016

FFQ, Food Frequency Questionnaire; RW, red wine; WW, white wine; DRW, dealcoholized RW; UCC, urothelial cell carcinoma; BCC, basal cell carcinoma; CRC, colorectal cancer; NHS, Nurses' Health Study.

Studies have shown an association between the increased consumption of alcohol (>3 glasses for men and >2 glasses for women) and an increased risk of many types of cancer (oral cavity, pharynx, larynx, oesophagus, liver, large intestine, and female breast) [47,48]. However, even moderate alcohol consumption was shown to result in an increased incidence of cancer by Klatsky et al. [49]. However, the authors note that it is likely that the self-reported alcohol consumption was underestimated, making it difficult to draw firm conclusions [49].

The role of lifetime alcohol consumption on invasive epithelial ovarian cancer risk was evaluated in a case–control study by Cook et al. [50]. With respect to wine, its consumption was associated with a lower risk of cancer compared to not drinking alcohol, while the association was stronger for red wine-only drinkers as compared to white wine drinkers. However, most women did drink both types of wine [50].

Concerning urothelial cell bladder cancer [51] and endometrial cancer risk [52], the EPIC cohort study did not show a significant association between wine consumption and these types of cancer. However, in the analysis by Fagherazzi et al. [53] on the role of alcohol on risk of adulthood breast cancer in 66,481 women from the French E3N-EPIC study showed that in the postmenopausal period, wine consumption increased the risk of breast cancer by 33% for more than two glasses per day of wine, as compared with non-drinkers. Risk of ER+/PR+ breast cancer subtypes was also influenced by high wine intake [53].

A prospective study [54] on alcohol consumption and risk of basal cell carcinoma with data from 167,765 women in the Nurses' Health Study (NHS) and NHS II and 43,697 men in the Health Professionals Follow-Up Study showed that increased wine consumption was associated with increased basal cell cancer risk after adjustment for sun exposure and other skin cancer risk factors [54], whereas no associations were observed between alcohol, red or white wine intake and risk of early-onset basal cell carcinoma in a case–control study [55]. Kubo et al. [56] examined the association between alcohol consumption and risk of melanoma and non-melanoma skin cancers in 59,575 white postmenopausal women enrolled in the Women's Health Initiative Observational Study. After 10 years of follow up, and after adjusting for confounders such as sun exposure and skin type, it was observed that those who consumed more than seven drinks per week were in greater risk of both melanoma and non-melanoma skin cancers. Additionally, compared to non-drinkers, higher lifetime alcohol consumption of white wine or liquor was associated with an increased hazard of both cancers [56]. White wine was also found to increase the risk of melanoma by 13% for every drink per day, after analysis of data from 21,052 participants of the Nurses' Health Study, Nurses' Health Study II, and The Health Professionals Follow-Up Study [57].

As far as head and neck cancer (HNC) risk and wine consumption is concerned, no significant associations were observed in the Netherlands Cohort Study [58]. In fact, wine consumption was largely, but not statistically significantly, inversely associated with overall risk of HNC, and HNC-subtypes [58]. Similarly, wine drinking was inversely, but not statistically significantly associated with lower risk of oesophageal adenocarcinoma [59].

In stage III colon cancer patients the role of alcohol consumption in prognosis and survival was investigated by Phipps et al. [60] with surprisingly positive outcomes. Alcohol consumption was not associated with colon cancer outcomes, yet mild to moderate red wine consumption at 1–30 glasses per month of red wine was associated with longer overall survival, disease-free survival, and time to recurrence [60,61]. Similar was the result of a study by Walter et al. [62] in 3121 colorectal cancer patients that were followed up for 4.8 years. Lifetime and one-year before diagnosis abstinence from wine were associated with poorer overall survival and cancer-specific survival [62]. In addition to this, Klarich et al. [63] showed that moderate alcohol consumption was associated with a non-significant risk for colorectal cancer, while in the context of the Mediterranean diet, in which wine is the most preferred alcoholic drink, moderate alcohol intake was associated with reduced risk for colorectal cancer [63].

In cancer patients, malnutrition and weight loss is a common finding that affects response to treatment, susceptibility to treatment-related adverse events, prognosis, and quality of life [64]. Common advice is to drink a glass of wine before meals to increase appetite. Jatoi et al. [65] tested this advice in advanced cancer patients with appetite loss in a randomised controlled trial. Here, 141 patients were randomised to receive either 2 glasses of white wine or an oral nutritional supplement for 3–4 weeks. There were no statistically significant differences concerning appetite between the two groups. Hence, white wine does not improve appetite or weight in this patient group [65].

3.6. Association between Wine Consumption and Mental Health

The role of wine on mental health has been investigated in four studies (Table 6).

Table 6. Association between wine consumption and mental health [60–65].

Clinical Sample	Dose/Type/Experimental Models	Main Results	References
12,326 individuals from the Swedish Twin Registry	Beer, wine, or 6 cL of 80-proof spirits/questionnaire	>12 g of alcohol per day may increase risk of dementia.	Handing E.P., et al., 2015
589 multi-ethnic community residents of New York aged ≥65 years	Beer, wine, or liquor/FFQ	Protective effect of wine on brain	Gu Y., et al., 2014
2613 participants, aged 43–70 years	Beer, wine (red, white and rosé), fortified wine and spirits/FFQ	Only moderate red wine consumption, associated with less strong cognitive decline	Nooyens A.C., et al., 2014
360 patients with early AD in New York, Boston, Baltimore and Paris	Alcohol intake/FFQ	Wine did not affect the rate of cognitive decline	Heymann D., et al., 2016
5505 high-risk middle aged and elderly men and women	Beer, wine, spirits/FFQ	2–7 units of wine per week, associated with 32% lower risk of depression, yet heavy drinking can increase the risk of depression	Gea A., et al., 2013
1572 adults living in southern Italy	Dietary intakes of polyphenols/questionnaire	Higher dietary intake of flavonoid may be inversely associated with depressive symptoms.	Godos J., et al., 2018

FFQ, Food Frequency Questionnaire; RW, red wine; WW, white wine.

The Swedish Twin Registry study, with 12,326 participants, showed that each additional gram of alcohol over 1.16 g per day from wine was associated with a 2% decreased risk of dementia, yet the

highest amount of alcohol intake from wine was associated with an increased dementia risk by 1% [66]. A cross-sectional study, used high-resolution structural MRI on 589 multi-ethnic community-dwelling elderly to assess the effect of alcohol intake and on imaging markers of brain structure. Those with light-to-moderate wine intake had larger total brain volume compared to non-drinkers, while there was a dose–response association between wine and total brain volume, indicating a protective effect of wine on brain [67].

Considering the rate of cognitive decline, its relationship with alcohol intake in middle age was investigated in the Doetinchem Cohort Study [68]. Here, 2613 participants aged 43–70 years at baseline were assessed every 5 years for 10 years. Red wine consumption was negatively associated with global cognitive function decline, memory and flexibility, with the best effect observed at 1.5 glasses of red wine per day. Since red wine was the only alcoholic beverage associated with cognitive decline indicates that non-alcoholic constituents in red wine may be responsible for this beneficial effect [68]. However, in a cohort of Alzheimer's disease patients, who were followed-up biannually for up to 19.28 years, wine did not affect the rate of cognitive decline [69].

The PREDIMED study investigated the association between wine consumption and depression in 5505 high-risk middle aged and elderly men and women, who were followed up to seven years. Moderate wine consumption of two to seven drinks per week was significantly associated with 32% lower risk of depression, yet heavy drinking can increase the risk of depression [70]. More recently Godos et al. [71] in their observational study with 1,572 adults, found a beneficial effect of polyphenols against depression. Specifically, wine consumption was negatively associated with depressive symptoms [71].

3.7. Association between Wine Consumption and Gut Flora

The effect of wine polyphenols on gut microbiota has been investigated in five studies in the last five years (Table 7).

Table 7. Association between wine consumption and gut flora [66–71].

Clinical Sample	Dose/Type	Duration	Main Results	References
41 volunteers, aged 20−65 years	250 mL/day RW	4 weeks	The microbial metabolic profile of faeces is significantly modified after moderate intake of red wine polyphenols	Munoz-Gonzalez I., et al., 2013
60 microbial phenolic metabolites in faecal samples	DRW: 272 mL, RW: 272 mL/, Gin: 100 mL/day	3 months	The microbial metabolic profile of faeces is significantly modified after moderate intake of red wine polyphenols	Jimenez-Giron A., et al., 2013
10 male volunteers, aged 45–50 years	RW (272 mL/day), DRW (272 mL/day), or gin (100 mL/day)	20 days	Chronic RW consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower plasma lipopolysaccharide (LPS) concentrations.	Clemente-Postigo M., et al., 2013
10 patients with metabolic syndrome and 10 healthy subjects	RW & DRW	30 days	Modulation of the gut microbiota by using red wine could be an effective strategy for managing metabolic diseases associated with obesity.	Moreno-Indias I., et al., 2016
38 volunteers, 55–67 years	100 mL per day RW/FFQ		Regular consumption of RW appears to be associated with a reduced serum lipoperoxidation in which the intestinal microbiota may be involved	Cuervo A., et al., 2015
41 healthy volunteers	250 mL of red wine per day	28 days	Consumption of red wine increased the global faecal microbial diversity	Barroso E., et al., 2017

FFQ, Food Frequencies Questionnaire; RW, red wine; WW, white wine; DRW, dealcoholized RW.

A randomised-controlled trial with 41 healthy volunteers investigated the changes in the microbial-derived phenolic metabolites of faeces, after consumption of 250 mL per day red wine for 4 weeks. Ten compounds, mainly benzoic and 4-hydroxyvaleric acids increased, after red wine

intake, while the total phenolic metabolites content was also increased. Hence, a different gut microbial capacity to metabolise wine polyphenols exists among people [72]. Similarly, another analysis by the same team showed that red wine and dealcoholized red wine change the content of eight phenolic acids probably derived from the catabolism of flavan-3-ols and anthocyanins, yet alcohol does not influence the formation of phenolic metabolites by the gut flora. Inter-individual differences were also observed [73].

The impact on lipopolysaccharides' concentrations after chronic and acute red wine intake in relation to high fat intake in middle-aged men was investigated by Clemente-Postigo et al. [74]. Ten middle-aged male volunteers were randomised to receive red wine, dealcoholized red wine, or gin for 20 days, and five adult men underwent a fat overload or a fat overload along with red wine, dealcoholized red wine, or gin. No significant differences in the change in lipopolysaccharide (LPS) or Lipopolysaccharide binding protein (LBP) concentrations with chronic red wine, dealcoholized red wine, or gin consumption were observed. *Bifidobacterium* and *Prevotella* amounts were significantly increased by red wine and were inversely correlated with LPS concentrations. There were no differences in postprandial serum LPS, LBP, or chylomicron LPS concentrations with acute red wine, dealcoholized red wine, or gin with a fatty meal. Hence, chronic red wine consumption increases *Bifidobacterium* and *Prevotella* amounts, which may have beneficial effects by leading to lower LPS concentrations [74]. Ten patients with metabolic syndrome and ten healthy subjects were included in a randomized, crossover, controlled trial. Participants consumed red wine and dealcoholized red wine for 30 days. The dominant bacterial composition did not differ significantly between the study groups after the two red wine intake periods. In the metabolic syndrome patients, red wine polyphenols significantly increased the number of faecal bifidobacteria and *Lactobacillus* and butyrate-producing bacteria at the expense of less desirable groups of bacteria such as LPS producers. The changes in gut microbiota in these patients could be responsible for the improvement in the metabolic syndrome markers. Modulation of the gut microbiota by using red wine could be an effective strategy for managing metabolic diseases associated with obesity [75].

The association between red wine intake, inflammation, and oxidative stress and faecal microbial populations was studied in 38 adult volunteers. Those who regularly consumed red wine at 100 mL per day had lower serum concentrations of malondialdehyde (MDA) and lower faecal levels of *Bifidobacterium coccoides* (*B. coccoides)*, Clostridium leptum (*C. leptum)*, *Bifidobacterium*, and *Lactobacillus*. A positive association between MDA levels and *B. coccoides* and *Lactobacillus* was also found. Thus, regular red wine intake can reduce serum lipoperoxidation, in a mechanism that involves the gut microbiota [76].

The influence of moderate red wine intake on the colonic microbiota was investigated in 15 healthy volunteers, who were classified into high, moderate, and low polyphenol metabolizers and were compared with five controls who did not drink wine. Consumption of red wine increased the global faecal microbial diversity [77].

4. Discussion

The most recent studies confirm the valuable role of moderate wine consumption, and especially red wine, on the prevention and treatment of chronic non-communicable diseases, such as cardiovascular disease [78–80], metabolic syndrome [81] and its components, cognitive decline [82,83], depression [84,85], and some cancers [86]. In the meantime, recent studies also highlight the beneficial role of red wine against oxidative stress [87] and on favour of a desirable gut bacteria [87].

More to the point, considering cardiovascular disease, studies continue to highlight the beneficial effect of wine [11,88,89]. Observational studies highlight a positive effect on cardiovascular events, mortality, and vascular inflammation in patients with established cardiovascular disease. Interventional studies show that moderate red wine intake lowers total and LDL cholesterol, as well as postprandial platelet aggregation and triglycerides in healthy participants. Considering patients with carotid atherosclerosis, patients with type 2 diabetes, patients in high cardiovascular disease (CVD)

risk, and patients with hypercholesterolemia, moderate red wine consumption is associated with better blood lipid control.

In addition to this, the risk of metabolic syndrome on healthy participants and elderly on high cardiovascular disease risk is lower than on those who do not drink alcohol, while interventional studies show improvements in glycaemic and lipid control have been observed in patients with MS, type 2 diabetes, and in those in high CVD risk. In patients with type 2 diabetes also show that moderate red wine intake can reduce the number of MS constituents. The protective role of red wine on metabolic syndrome has been stated in other studies, highlighting the role of ethanol and polyphenols in modulating the endothelial nitric oxide synthase [90], while resveratrol may have protective effects against the MetS via AMP-activated protein kinase and by promoting mitochondria biogenesis [81], in addition to acting as an activator of the NAD(+)-dependent deacetylases sirtuins [81], which have been shown to extend life in animal models [91,92] and prevent insulin resistance and metabolic derangement [81].

Considering blood pressure, recent interventional studies show inconclusive results. An increase on red wine intake by women does increase blood pressure as compared to non-alcoholic wine, while two glasses of red wine increase heart rate and decrease arterial compliance after consumption, and three glasses at first decrease and then increase blood pressure (BP). In patients with diabetes, two to three glasses of red wine also increase BP (which is later lowered), yet long-term red wine intake at 1 glass with dinner per day does not influence BP in this patient population. However, in patients with mild hypertension a decrease in BP has been observed, which is attributed to the catechines and procyanidins in wine. Carolo et al. [93] highlighted that moderate wine consumption, in addition to the Mediterranean diet, may help manage hypertension [93], while Garcia-Conesa et al. [80] in their meta-analysis found that blood pressure was significantly reduced by red wine anthocyanins [80].

The association between wine consumption and risk of cancer is inconclusive, as studies show both positive and negative associations between alcohol, wine, and different types of cancer [94–96]. Recent studies confirm that moderate wine intake has been associated with lower risk of epithelial ovarian cancer, but higher risk of breast cancer [97,98], melanoma, and other skin cancers [57]. Additionally, recent studies show a possibly higher risk of basal cell carcinoma, yet prior evidence is inconclusive [54,99]. However, in stage III cancer patients low wine intake has been associated with longer overall survival, disease free survival, and longer time to relapse.

Considering mental health, moderate wine intake has been associated with lower risk of cognitive impairment, and greater total brain volume, whereas high amounts have been associated with greater risk of cognitive impairment. Additionally, observational studies have shown a negative association between cognitive decline and memory, attributed to the non-alcoholic part of red wine, yet no associations have been observed in Alzheimer disease patients. Orgogozo et al. [100] in their retrospective study with 3777 elderly also found an inverse association between wine intake and incident dementia, while Pasinetti [101], Pinder et al. [102] and Granzotto et al. [103] highlighted the beneficial role of wine polyphenols in the prevention of Alzheimer's disease.

Moderate wine consumption also lowers the risk of depression in middle aged and elderly people, as well as in patients with chronic heart failure. Gea et al. apart from their study on wine and depression for the PREDIMED study [70] have also shown a positive effect of moderate alcohol intake against depression in women, in the Seguimiento University of Navarra (SUN) project [104].

As far as gut microbiome is concerned, red wine polyphenols exert prebiotic effect, while augmenting the gut flora diversity. Studies show lower lipopolysaccharide and MDA concentrations after red wine consumption, while MS patients have a more desirable microbiome after red wine consumption. Such positive effects have also been observed in prior studies [105,106].

The beneficial role of red wine has been attributed to its phytochemical compounds, as highlighted by clinical trials, where the effect of red wine has been compared to that of white wine, non-alcoholic wine, other alcoholic drinks, and water.

However, the beneficial effects of moderate alcohol consumption may not be applicable to all people, as individual characteristics and population groups may not benefit from alcohol consumption. Pregnant women belong to a group which may not benefit from alcohol intake. While alcohol concentrations in breast milk are very similar to those in maternal blood and although alcohol may cause a temporary decrease in milk yield, data on alcohol consumption during lactation are contradictory [107]. Regarding children, alcohol-related studies are scarce, as can be assessed either theoretically either by recall from adolescents or by research with children, which is difficult as the parents' consensus reluctance on the participation of children in alcohol-related research limits this possibility. Studies have shown that early onset of alcohol consumption results in many unfavourable outcomes in both adolescence and adulthood, such as absence from classes, violent behaviour, and depression symptoms [108,109].

As the gastrointestinal tract is the first to be affected by our dietary choices, the intake of alcohol is a major factor in the encumbrance of gastrointestinal diseases. For this reason, it is encouraged to avoid alcohol consumption, although some studies report an unclear impact on the symptoms of the gastrointestinal tract from the different forms of intake [110–112].

Another condition to be considered is alcohol consumption in liver diseases, as studies report unclear findings regarding low to moderate consumption [113], with some studies reporting that alcohol can suppress the activity of non-alcoholic steatohepatitis by reducing the expression levels of genes involved in the immune response [114].

Another important issue is the alcohol–drug interaction, as the metabolism of a drug may increase or decrease, resulting in a change in blood levels (bioavailability) and drug half-life. Some of the classes of drugs that are affected by alcohol consumption are commonly used, for instance antihypertensives, antibiotics, antihistamines, opioid analgesics, and others [115].

Although moderate alcohol consumption as part of a healthy diet and combined with regular physical activity can be associated with beneficial health effects, high consumption and cumulative action are associated with a large number of harmful effects, affecting organs, systems and behaviour. Also, high consumption and cumulative action increases mortality, cardiovascular function, metabolic profile, and organ function, and thus initiation of alcohol consumption cannot be recommended to non-consumers [13]. As alcohol consumption has increased in recent years, the World Health Organization encourages strategies regarding the reduction of the alcohol content of alcoholic beverages in order to reduce its harmful effects [112].

It is important to highlight the limitations of the included studies. The evaluated studies included a large proportion of observational studies involving either groups of subjects or participants comparing their behaviour, diet, substance use, and exercise habits and/or based on characteristics such as sex and ethnicity. In these studies, the correlations between the potential risk factors that are beneficial or harmful from the consumption of wine and their disease outcomes over time can be identified but do not imply causation.

Another limitation concerns the gathering of information using a questionnaire and the confounding factors arising from it, such as memory recall ability, closed-ended questions, the presence or not of the researcher, and the fact that respondents often respond to what they believe the correct answer is. Furthermore, in the randomized controlled trials, heterogeneity concerning the follow-up period was observed, as well as the number of participants and the age groups.

In addition to this, most observational studies do not differentiate between white and red wine, hence, it is not possible to distinguish the part of the wine exerts beneficial role, while interventional trials study various wine amounts, and most times different amounts between men and women, and the study periods also vary, from acute consumption to weeks- and years-long consumption.

Additionally, wines differ in their composition from place to place and grape variety, and may also differ because of differences in soil, weather, climate, harvesting, wine-making, ageing, bioavailability of bioactive components, and consumed quantity. Furthermore, as supported by

evidence, individual characteristics such as gut flora and genetics do play a role in the benefit exerted from wine consumption.

5. Conclusions

In conclusion, moderate wine intake at 1–2 glasses per day according to the guidelines [15] as part of the Mediterranean diet has been positively associated with positive effects on human health promotion and disease prevention, as well as disease prognosis. Despite this, non-drinkers should not be encouraged to initiate consumption of alcohol, while population groups that are not likely to benefit from alcohol (or wine) intake should be discouraged from drinking alcohol.

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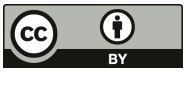

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