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Anticancer Effects of Grape Seed Extract on Human Cancers: A Review

Dinicola S[1], Cucina A[2], Antonacci D[3] and Bizzarri M[4*]

[1]Department of Clinical and Molecular Medicine, La Sapienza University, Rome, Italy
[2]Department of Surgery "Pietro Valdoni," La Sapienza University, Rome, Italy
[3]Consiglio per la ricerca e la sperimentazione in agricoltura, Unità di ricerca UTV, Turi BA, Italy
[4]Department of Experimental Medicine, La Sapienza University, Rome, Italy

Abstract

Grape seed extract (GSE) is a complex mixture of several compounds, mostly represented by polyphenols and phenolic acids. Their consumption is safe and is recognized to exert several and meaningful health benefits. In particular, grape-related anti-tumoral activity encompasses a wide array of biological mechanisms and cellular targets, eventually leading to inhibition of cell growth and to enhanced apoptosis in several cancer cell lines, including lung, colon, breast, bladder, leukemia and prostate tumors. Those effects are likely modulated at the molecular level through selectively modulating the redox balance and displaying anti-oxidant as well as pro-oxidant actions, according to the specific context. GSE-related anti-cancer activity mostly relies on the induced increase in reactive oxygen species, followed by the orchestrated down- and up-regulation of several key-molecular pathways, including MAPK kinases, PI3K/Akt, NF-kB, cytoskeleton proteins and metalloproteinases. Promising results obtained in vitro as well as on animal studies suggest that GSE may have a great relevance as source of potential new pharmacological molecules, and could represent an important opportunity for clinical research.

Keywords: GSE; Apoptosis; Chemoprevention; Oxidation

Introduction

Grape seeds and fruits

Cancer is among the leading cause of death in the Western world and its incidence is rising sharply in the developing countries too. By no doubt, that trend can be likely ascribed to the world-wide adoption of western dietary habits, characterized by high saturated fat diet, low intake of fresh vegetables and fruits, with reduced assumption of polyphenolic-rich foods (like green tea, soy and grape seeds) [1]. On the contrary, high and regular consumption of polyphenolic-rich foods has proven to significantly reduce the incidence of breast, lung, prostate and gastro-intestinal human cancers [2]. Among those foods, a prominent role is undoubtedly sustained by grapes and grape-related aliment and beverages.

From time immemorial grapes have been used both for medicinal and nourishment purposes, chiefly in Greece and in Italy. Grapes (*Vitis vinifera*) have been heralded for their medicinal and nutritional value for thousands of years: Egyptians ate grapes at least 6,000 years ago, and several ancient Greek philosophers praised the healing power of grapes, usually in the form of wine. The role that the grape has in the food culture of the Mediterranean countries is comparable only to that played by tea in among the peoples of Asia, indeed. An impressive body of the current scientific literature supports the health benefits claimed by the medical tradition. Several epidemiological studies have associated the consumption of grapes, wine, and grape juice with a wide variety of health-promoting effects, particularly the reduced risk of cancer and cardiovascular diseases [3-6]. It is worth of mentioning that a significant linear decrease in risk of lung cancer associated with consumption of red wine among ever-smokers has been recorded by a multiethnic cohort study involving more than 80,000 men: consumption of 1-2 cup of wine reduces the risk of lung cancer of approximately 60%. A similar trend has been observed by other studies [7-10]. Interestingly, a similar pattern has been recorded by epidemiological studies performed on Green Tea [11-14].

Tea and grape have different chemical composition [15]. Yet, many GSE components (epigallo-catechins, procyanidins, flavonoids) are also found in Green Tea, and they may well account for the widely recognized beneficial effects of tea consumption. However, even if a consistent overlap has been observed in between the biological properties of both mixtures, yet extracts from grapes and tea differ significantly in their effectiveness, given that when they are simultaneously added to cancer cells, a synergistic, significant effect can be observed [16]. Yet, the beneficial properties of both tea and grape (or grape derived food products), are believed to be related to their polyphenolic content [17,18]; and, by no doubt, grapes constitute one of the major sources of phenolic compounds among fruits [19].

Grape seed composition

Grape seed composition differs significantly in between different cultivars [20-23], namely when white versus red grapes are considered. Yet, those differences reflect not only genetic variability, but also highlight the impact of vineyard treatments, ripeness grade [24,25], irrigation strategy [26,27] and nitrogen fertilization [28]. Even within seeds obtained from the same cultivar a significant variability in chemical composition has been recorded, and such a result may be likely ascribed to differences in the extraction method [29-31]. In addition, several environmental and biological factors, such as hyperopic, light, drought, high salinity, cold, metal ions, pollutants, xenobiotics, toxins, reoxygenation after anoxia, experimental manipulations, pathogenic infection and ageing of plants may affect yields and seed quality, mainly by inducing oxidative stress [32,33]. Nonetheless, plant cells have a wide array of detoxifying enzymes and pharmacologically active, anti-oxidant compounds that scavenge Reactive Oxygen Species (ROS), participate in seed survival, and may hence display relevant pharmacological activities [34]. Besides some minor components, main grape seed constituents are represented by polyphenols,

***Corresponding author:** Mariano Bizzarri, Department of Experimental Medicine, La Sapienza University, Rome, Italy, Tel: +39(0)649766606; Fax: +39(0)649766897; E-mail: mariano.bizzarri@uniroma1.it

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phenolic and hydroxy-benzoic acids. Stylbenes (trans-resveratrol) as well, are occasionally found, even if in a few varieties [35]. Polyphenols (Flavonoids) is a collective noun given to several classes of structurally similar compounds, having a common C6-C3-C6 flavone skeleton in which the three-carbon bridge between the phenyl groups is commonly cyclized with oxygen. Flavonoids include several classes of compounds: Flavones (luteolin), Flavan-3-ols (catechins, epicatechins, epigallo-catechins, epigallocatechin-3-gallate, procyanidins), Flavanones (neringein), and Flavonols (quercetin, rutin, kaempherol) and Anthocyanins (Table 1) [36]. Each class differs from the other according to the degree of unsaturation and oxidation of the three-carbon segment [37]. Flavonoids are usually present in nature as glycosides: the sugar moiety attached to the flavonoid structure affects ease of absorption from the intestinal tract and the bioavailability of the compound. Yet, glycosylation lessens the reactivity of flavonoids against free radicals and slow-down their intestinal absorption [38]. Grape seeds have higher content of both phenolic acids and flavonoids (where they account for 60-70% of dry extract) [39] than grape skin and whole grape extract, meanwhile resveratrol and anthocyanidins are more abundant in the latter two extracts [40].

Several individual grape seed components (Figure 1) have been demonstrated to display relevant chemical and biological functions, such as antioxidant [41], anti-inflammatory [42], inhibition of platelet aggregation [43], antimicrobial [44], and "anti-aging" activities [45]. Those properties have been found to be directly associated to the total polyphenolic content [46,47] and specifically ascribed to the activity of the more effective components, among which ellagic [48] and gallic acid [49], epigallocatechin-3-gallate [50], procyanidins [51] and quercetin [52] are by far the most important. Gallic acid, procyanidins and epigallo-catechins overall account for about 80-90% of dry extract [53], and medical properties of grape seeds are generally referred to those molecules, indeed [54]. Yet, the contribution of other active, even less represented molecules cannot be excluded, given that some biological functions seem to be synergistically afforded by interactions among the different components [55]. Namely, the well-known anti-oxidant effects exerted by GSE, can only barely be explained by the sum of the anti-oxidant activities of each individual component. Indeed, correlation analysis showed that none of the identified polyphenols had a strong correlation with protection from ROS [56]. Thus, it seems that there may be a synergism between polyphenols and/or between polyphenols and phenolic acids and other phytochemicals. Similarly, even if anticancer effects are generally thought to be exerted mainly by procyanidins and epigallo-catechin-3-gallate (EGCG), again the overall GSE anticancer effect is higher than that obtained by the sum of each individual component [57].

Dual Effects of Grape Seed Extract: Anti-Oxidant and Pro-Oxidant Activities

GSE, as well as many of its individual components have demonstrated both *in vitro* and *in vivo* to prevent carcinogenesis [58-60], to inhibit cancer cell proliferation and to enhance cancer cells apoptosis, often reaching efficiency rates equal or greater than that achieved by conventional drugs. Yet, the by far most relevant feature of GSE is the dual role displayed in normal and cancerous cells. Grape and tea extract are safe, even at the highest concentrations [61-63], and exert a wide array of protective actions. Indeed, GSE and many phytochemicals inhibit apoptotic process and display a strong anti-oxidant effect on normal cells, meanwhile the opposite is true for cancer cells: neither growth inhibition nor apoptosis have been noticed in normal cells even at higher doses of GSE [64]. How and why that paradoxical behaviour occur is still a matter of investigation, even if an increasing body of evidence suggest that a possible explanation may be provided by the dual role exerted on the intracellular ROS formation.

GSE displays pro-oxidant effects on cancer cells

Antioxidant activities of GSE and grape phenolic compounds (mainly resveratrol and procyanidins), have been extensively investigated *in vitro* and *in vivo* [65]. GSE possesses strong free radical scavenging activity [66], prevents ROS-induced DNA damage [67], and displays a relevant chelating effect on transition metal ions, thus reducing lipid peroxidation [68]. Those effects have been deemed even more potent than known antioxidants such as vitamin E and ascorbic acid [69]. Some studies have reported an enhancing effect of GSE or of its polyphenolic constituents, on several anti-oxidant enzymes as glutathione (GSH) [70], super-oxide dismutase (SOD) [71], catalase [72] and other detoxifying/antioxidant enzymes [73]. GSE-induced antioxidant enzyme expression is associated with activation of the redox-sensitive transcription factor nuclear factor erythroid-2 p45 (NF-E2)-related factor (Nrf2), through its interaction with the antioxidant-response element (ARE) or the electrophile-responsive element (EpRE) [74,75]. Indeed, Nrf2 plays a key role in up-regulation of many phase II antioxidant/detoxifying enzymes, including glutathione peroxidase (GPx), glutamate cysteine ligase (GCL), glutathione S-transferase (GST), SOD, and NADPH/quinone oxidoreductase 1 (NQO1) [76].

In vivo, dietary supplementation of GSE was shown to reduce oxidative stress and improve the glutathione/oxidized glutathione ratio, as well as the total antioxidant in a double-blinded randomized crossover human trial [77]. Though those results have been often confirmed [78], other studies have been unable to do so, showing that GSE exhibits either only a moderate or negligible antioxidant effect [79,80].

Oxidative stress, resulting from enhanced production of ROS overcoming the cellular antioxidant defence, is a key phenomenon in chronic degenerative diseases (diabetes mellilitus, cardiovascular illness, cancer) [81,82]. ROS participate in triggering the apoptotic process, as programmed cell death is tightly regulated by the oxidative environment [83]. Dietary GSE strongly reduces rat mucosal apoptosis via modulation of both mitochondrial and cytosolic antioxidant enzyme systems together

Hydroxybenzoic and Phenolic Aids	Polyphenols (Flavonoids)						Still Benes
Ellagic acid*	Flavones Luteolin* Diosmetin Apigenin Chrysin wogonin	Flava-3-ols Catechin* Edpicatechin* Epigalloctechin* Epigalloctechin-3-gallete* B1,B2,B3,B4 procy-anidins*	Flavanones Naringenin* Hesperidin Eriodictyol	Flavonds Quercetin* Myricetin* Kaemoferol* Rutin*	Isoflavones Genistein Daidzein	Anthocyamins Delphinidin Pelargonidin malvidin cyanidin petunidin	
Gallic acid*							Reveratrol*
Vanillic acid*							Trans-reveratrol*
Caffeic acid*							
Coumaric acid*							
Ferulic acid*							
Gentistic acid*							

Table 1: Grape seed composition. Principal classes of polyphenols and phenolic acids; re[illegible]s evidence compounds found in grape seeds by different analytical studies [15,19-22,28-30,39-40].

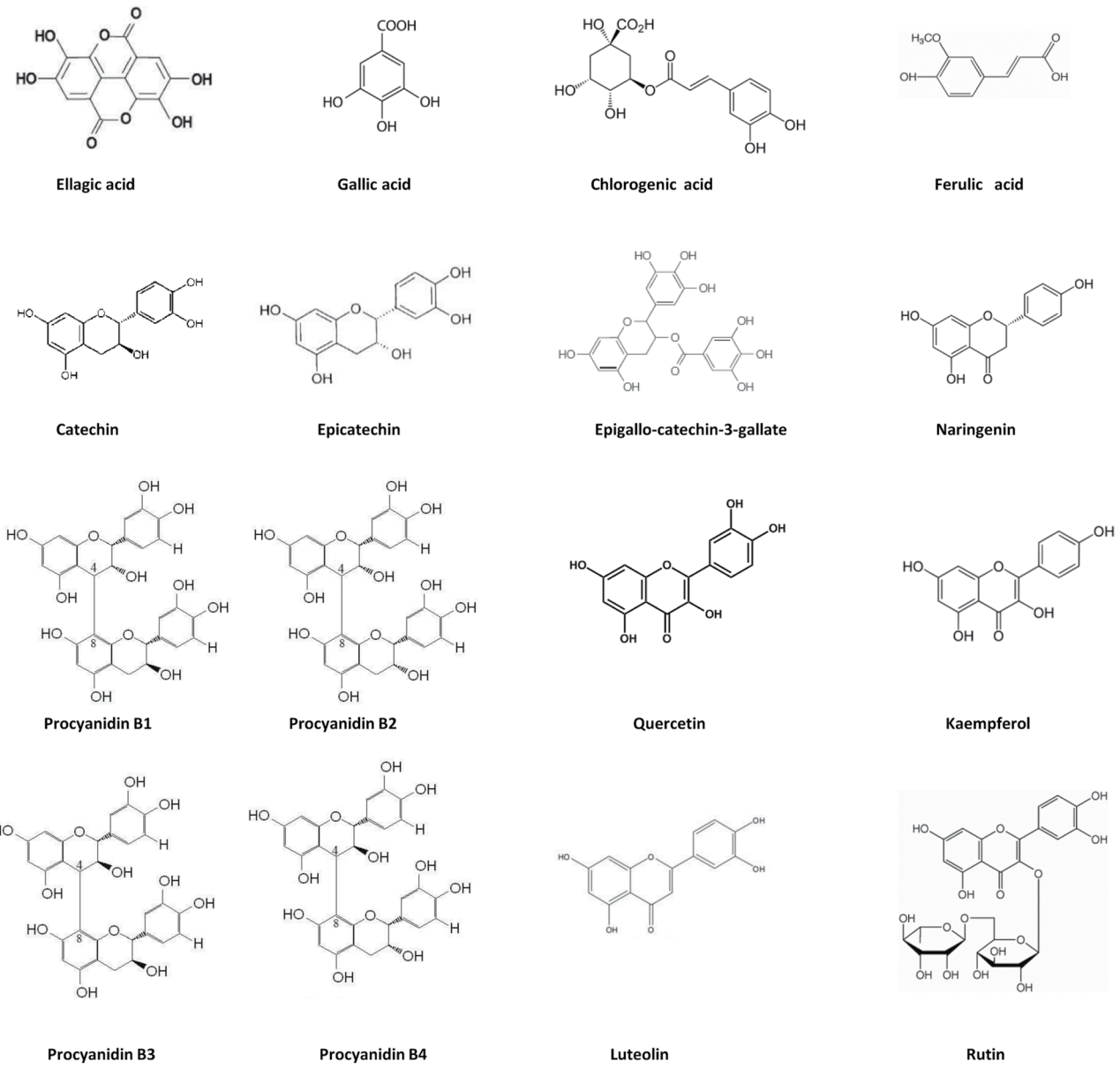

Figure 1: Chemical structures of the principal GSE components.

with an increase in cellular GSH, thus protecting normal colonic mucosa from ROS injury [58,84]. Given that GSE exerts a protective anti-oxidant effect in normal cells exhibiting deficiency of catalase activity or glutathione level, it can be hypothesized that grape polyphenols participate in controlling intracellular peroxide production [85]. Hence, anti-oxidant properties of GSE treatment may efficiently counteract the onset of ROS-dependent disease, as documented by several studies [86]. Yet, despite the popular version diffused by mass-media, it is hardly conceivable that GSE or polyphenols may exert a significant effect against cancer development by displaying anti-oxidant actions [87].

Indeed, several studies have reported that GSE in cancer cells paradoxically enhances ROS production in a significant manner. GSE and many polyphenolic compounds induce a relevant increase in ROS and in superoxide radical generation, at both the cytosolic and mitochondrial site, that could eventually lead to GSH depletion [88]. It is worth noting that GSE does not induce hydroxy peroxide (H_2O_2) increase, thus evidencing a deficiency in SOD activity, at least in the cancer cell lines studied. Indeed, in SOD-deficient cells, GSE treatment induce ROS-mediated cytotoxicity, evidencing that GSE-dependent increase in ROS activity is not efficiently counteracted by SOD-dependent transformation in hydroxy peroxide, leading to GSH depletion, cellular damage, and increased apoptosis [89]. Moreover, pro-oxidant effects of GSE are enhanced in cells lacking SOD activity [90], meanwhile co-exposures of polyphenols-treated cancer cells with SOD largely prevented ROS formation and DNA damage [91]. Considering that the oxidant-dependent toxicity of polyphenols is efficiently rescued by co-treatment with SOD, but not with catalase, it is unlikely that flavonoids-related pro-oxidant effects could be mediated

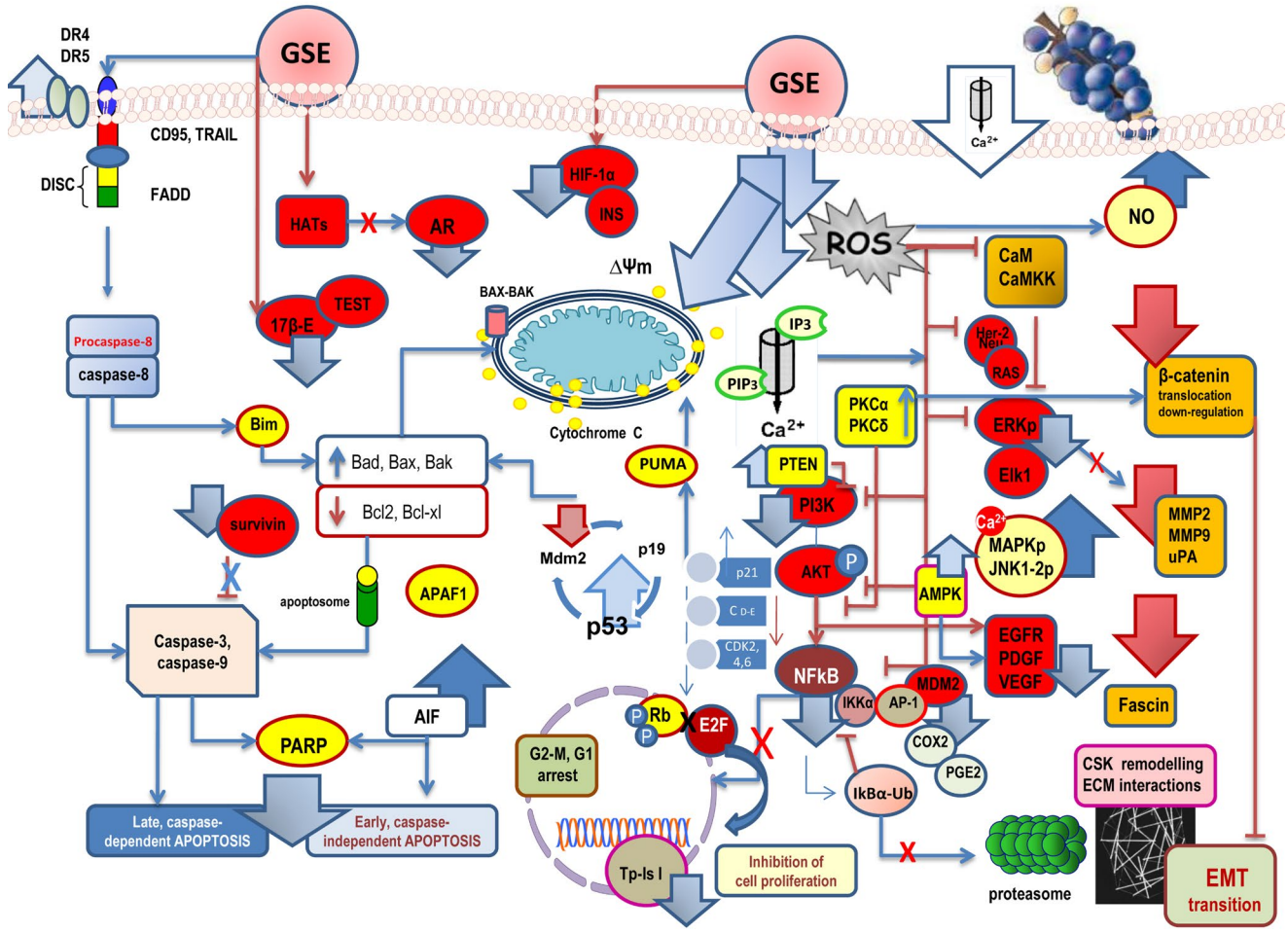

Figure 2: Molecular mechanisms of GSE interactions. GSE interacts with many cellular biochemical and genetic pathways, through which cell proliferation, cytoskeleton rearrangement, apoptosis and cell differentiation are modulated. A pivotal key-step is supported by the increase in ROS formation induced in cancer cells by GSE through the selective regulation of the redox balance. Acronymous: DISC, death-inducing signaling complex; FADD, Fas associated death domain; transmembrane death receptors, DR4, DR5; tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); Insulin, INS; cAMP response element-binding protein (CREB); poly-ADP-ribose polymerase, PARP; FOXO1, forkhead box O1; histone androgen acetyltransferase, HATs; Apoptosis Inducing factor, AIF; Apoptotic protease activating factor 1, APAF; Hypoxia-inducible factor 1-alpha, HIF-α; testosterone, TEST; Androgen receptor, AR; Inositol-3-phospgate, IP_3; Phosphatydyl-Inositol-3-phosphate, PIP_3; Calmodulin, CaM; Calmodulin kinase, CaMKK; PKC, protein kinase C; p53 upregulated modulator of apoptosis, PUMA; phosphatase and tensin homologue deleted on chromosome ten, PTEN; nuclear factor kappa B, NF-kB; inhibitor of kappa B, IK-b (comprising the subunits IKKα,β,γ; Mouse double minute 2 homolog, MDM2; activator protein 1, AP-1; Nitric oxide, NO; urokinase-type plasminogen activator, uPA; matrix metalloproteinases,MMP^{2-9}; Topoisomerase-I, Tp-Is 1; Prostaglandin-endoperoxide synthase 2 or cyclooxygenase-2, COX-2; prostaglandin E2, PGE2; 5' AMP-activated protein kinase, AMPK; ETS domain-containing protein, Elk-1; cyclin, C; cyclin-dependent kinases, CDK; ubiquitination complex, Ub; cytoskeleton, CSK; extra-cellular matrix, ECM; epithelial-mesenchymal transition, EMT.

by H_2O_2 but rather by the super oxide anion O_2.- , whose activity is unaffected by catalase, indeed [92]. This specific feature is displayed by the overall extract from grape seed, as pro-oxidant effects triggered by individual polyphenols – obtained from tea or grapes – can be significantly inhibited by adding catalase in the culture medium [93,94]. Several indirect experimental evidences support these observations. Indeed, cell culture medium amended with either green tea or with red wine inhibited proliferation of rat pheochromocytoma PC12 cells: both supplementation generated ROS, and the addition of catalase completely abolished the anti-proliferative effects of green tea, but only partially reduced that of red wine [95]. Apparently, this result would suggest that ROS accounted for the total cytotoxic effect of green tea, but only partially for that of red wine. As catalase may detoxify only H_2O_2, it should be hypothesized that anticancer actions of GSE may be exerted through the increase of different reactive oxygen species, among which H_2O_2 represent only a negligible fraction. In addition, polyphenols-treated cancer cells exhibit activation of both early ROS-dependent and late-ROS-independent genes associated with cell cycle modulation and apoptosis. ROS-dependent genes activated after polyphenols addiction have been identified as early response or biphasic genes [96]. By suppressing H_2O_2 co-amending cell culture with catalase, only early ROS-dependent apoptosis is abolished, whereas late apoptosis is retarded, as apoptotic cell death still occurred but after a delay of 24 hours. That biphasic-effect on cell apoptosis has been reported by many other studies performed on tea polyphenols [97,98], indicating that polyphenols increase ROS, both sensitive (as H_2O_2) and insensitive to catalase [99] (as superoxide anions) [100], or, alternatively, they may activate an unrelated

ROS-independent apoptotic pathway.

Furthermore, several studies have noticed greater depletions of intracellular GSH in cancer than in normal cells upon their exposures to polyphenols, including grape seed and tea polyphenols extract [101]; in turn, by blocking the recycling of intracellular GSH with an irreversible inhibitor of glutathione reductase, antiproliferative effects of pholyphenols are greatly potentiated [102]. On the contrary, by adding N-acetyl-cysteine - a precursor in the synthesis of glutathione – generation of intracellular ROS was strongly lessened upon exposure of cancer cells to GSE or tea-derived polyphenols [103-105]. Flavonoids-induced reduction in GSH availability is however significantly dependent on the dose, given that very low, non-toxic concentrations of quercetin enhance the synthesis of GSH in monkey cancer kidney cells through up regulation of γ-glutamyl-cysteine synthetase, whereas exposure of cells to high concentrations of GSE or grape/tea polyphenols led to elevated levels of ROS, which quickly depleted GSH stores and thereby increase cellular susceptibility to oxidative free radical attack, resulting in cell death by either apoptosis and/or necrosis [106,107].

These results may contribute to explain the aforementioned paradoxical behaviour of GSE, highlighting how the pro-oxidant or anti-oxidant effect is context-dependent, as it is shaped by the overall architecture of the redox balance. It should be outlined that such effects have been recorded only for high doses of bioactive compounds, given that a very low GSE concentration (in the range of 1-10 nm) prevents ROS-induced oxidative cell damage, restores intracellular glutathione content, and ameliorates mitochondria-mediated and death receptor-mediated apoptosis in both normal and cancer liver cells [108].

Those effects have been recorded in several cancer lines amended with GSE by ours and other groups [64, 103,109-111], and noticed also when using single polyphenolic molecules [112-114]. It is worth noting that the pro-oxidant effect is a very early event (occurring after 5-30 minutes after GSE supplementation), and it happens well before the subsequent onset of apoptosis and cell cycle inhibition. Pre-treatment with N-acetyl-cysteine (NAC) or other ROS-scavenging molecules abolishes almost completely GSE-dependent anticancer effects, and such results indicate that oxidative stress represents a meaningful initiating step. Therefore, GSE-induced oxidative stress in cancer cells should be considered the key-event, preceding the complex molecular cascade leading to GSE-dependent cancer inhibition.

Ros, mitochondrial potential and calcium

GSE, as well as many grape polyphenols and phenolic acids, have been shown to induce significant inhibition of cell proliferation and to enhance apoptosis in several cancer cell lines. Those effects occur at both low and high GSE concentration, the necrotic processes becoming more evident for the highest GSE doses. Such effects have been recently demonstrated to be dependent on ROS formation, occurring early after GSE administration in lung, bladder and colon cancer cells [103,115]. Concomitantly to ROS enhanced formation, the mitochondrial membrane potential was significantly reduced, dose and time-dependently in GSE treated cancer cells [116]. Similar findings have been also reported by adding tea polyphenols to a wide array of cancer cell lines [117]. Those effects were long-lasting, as the decrease in mitochondrial potential still remains after 3-6 hours [118,119]. The mitochondrial transmembrane potential is often used as an indicator of cellular viability and metabolic activity, and its disruption has been involved in a variety of apoptotic phenomena [120].

Moreover, mitochondria have also been implicated in ROS generation during apoptosis. Indeed, reduced mitochondrial membrane potential has recently been shown to lead to increased generation of ROS and apopt[illegible] [121]. Furthermore, mitochondria are central players in cellular Ca^{2+} signalling given that they contribute in shaping and buffering cellular Ca^{2+} signals [122,123]. It is widely recognised that Ca^{2+} displays growth inhibiting and differentiation-promoting activities in a variety of normal and malignant epithelial cells. We have reported [103] that intracellular Ca^{2+} rapidly increased after the addition of GSE to cell cultures. This effect might be due to the mobilisation of intracellular Ca^{2+} stores, or to the influx of extracellular Ca^{2+}. In order to address these issues, Caco-2 colon cancer cells were incubated in a Ca^{2+}-free medium containing the Ca^{2+} chelator EGTA, before addiction of GSE obtained from different grape cultivars (*Red Globe, Italia and Palieri*). Addition of EGTA does not modify intracellular concentration of Ca^{2+} in *Red Globe*-treated cells, indicating that modification in intracellular Ca^{2+} was tightly dependent on extracellular Ca^{2+} influx in this very case. However, addition of EGTA to the medium supplemented with GSE obtained from *Italia* and *Palieri* cultivars, slig[illegible]reduced but did not completely inhibit t[illegible]crease observed in C[illegible]ntracellular levels, thus demonstrating that [illegible]release in these specific cases is largely due to the depletion of intracellular Ca^{2+} stores. Yet, addition EGTA abolished almost completely GES-induced apoptosis on colon cancer cells as well as mitochondrial depolarisation, thus suggesting the two phenomena are entrenched. Further addition of NAC did not modify significantly those results, suggesting that ROS-induced Ca^{2+} release is a mandatory step in anticancer effects triggered by GSE. As previously suggested [124], those data outline a crosstalk signalling in between Ca^{2+} and ROS: ROS may regulate the activity of Ca^{2+}-activated channels and, at the same time, increased Ca^{2+} levels could reinforce ATP synthesis-induced ROS generation. GSE-induced elevation in intracellular calcium levels is also associated to a dramatic down-regulation of Calmodulin A (CaM) in breast cancer cells [125]. CaM binds to calcium and hence activates several pathway involved in cancer progression, and increased levels of CaM have been found in cancer cells [126]. However, uncoupled Ca^{2+} activates the RAF/MEK/ERK pathway and promotes phosphorylation of MAPKp38 and JNK, eventually leading to over-expression of p53 [127].

GSE and Cell-Cyle Modulation

Disruption of the normal regulation of cell-cycle progression and division are important events in the development of cancer. Several proteins are known to regulate the timing of the events in the cell cycle. Major control switches of the cell cycle are the cyclins and the cyclin-dependent kinases (CDKs). GSE and many of its constituents (chiefly EGCG and resveratrol) [128] have been demonstrated to exert their antiproliferative effects on leukemia [129],ovary [130], lung [131,132] , head and neck [133], prostate [51,134], breast [135,136] and colon cancer, both *in vivo* and *in vitro* [137-139]. It is worth noting that, as previously outlined, either native GSE or Tea extract, display a significant greater anti-proliferative effect than isolated compounds or synthetic mixtures [140]. GSE treatment resulted in a marked reduction in the expression levels of CDK2, CDK4 and CDK6 [141]. Similarly, a marked reduction in cyclins D1, D2 and E, and an increase expression of negative regulators of the cell cycle (Cdki, such as p21 and p27) were observed after GSE treatment, eventually inducing a dramatic inhibition of cell growth, and a consequent cell cycle arrest in G1, S or G2/M phase [142]. The antiproliferative GSE-based effect involves several molecular targets, including up-regulation of Rb phosphorylation and down-regulation of E2F, through modulation of the EGFR-ERK1/2 pathway [133].

Eventually, those signals converge and activate cyclins, which bind to Cdki to induce cell cycle progression towards S phase. CDKs activity is required to allow cancer progression, and their functions are tightly

regulated by Cdki. The increased expressions of Cdki together with decreased expression of cyclins (namely Cyclin-1) [143], and CDKs on GSE-treated cancer cells suggest that GSE might be effective as a chemotherapeutic agent for the treatment of a wide array of tumors. Those effects are likely to involve GSE-mediated inhibition of PI3K/Akt pathway, down-regulation of the epidermal growth factor receptor (EGFR) [144], and interference with NF-kB activity (Figure. 2).

Pro-Apoptotic Effects of GSE

GSE and MAPK kinases

The extensive investigations with the GSE have identified various molecular targets involved in GSE-mediated cancer cell apoptosis.

The PI3K/Akt pathway plays a pivotal role in mammalian cell survival signaling and has been shown to be activated in various cancers [145]. Indeed, phosphorylated PI3K and Akt are thought to be key factors in modulating down-stream kinases activation and NF-kB-dependent pathways. It is worth of noting that grape and tea polyphenols [146], as well as GSE, have been shown to decrease the PI3K levels and Akt phosphorylation, even enhancing proteasome degradation of Akt in several cancer cell lines [147]. Down-regulation of the phosphorylated form of PI3K is a key event in Akt regulation: Akt binds to phosphatidyl-inositol-3-phosphate (PIP_3), and PI3K induces its phosphorylation at the carboxy-terminal of Ser^{473} residue. PI3K is negatively regulated by the phosphorylated form of phosphatase and tensin homologue deleted on chromosome ten (PTEN), a lipid phosphatase that catalyzes the dephosphorylation of PIP3 and thus inhibit PI3K/Akt phosphorylation [148]. Absence of PTEN strongly correlates with activation of PI3K/Akt in tumour cell lines [149], whereas GSE significantly decreased PTEN phosphorylation, and thereby increased its negative regulation on the PI3-K pathway [150]. Phosphorylated Akt may in turn activate survival pathways by directly phosphorylating specific targets. Indeed, Akt negatively regulates factors that promote the expression of death genes (Bad) [151] and positively regulates antiapoptotic factors (Bcl-2, CREB) [152,153] and pro-survival genes (FHKR, NFkB) [154,155]. GSE significantly inhibited Akt-dependent FKHR phosphorylation in Caco-2 cells, thus leading to FHKR proteins residing predominantly in the nucleus where they are able to promote transcription of pro-apoptotic target genes such as Fas-L and Bim through specific DNA elements in their promoters. In addition, GSE suppresses Akt-related effects on CREB, NFkB [135], BAD and Bcl-2, thus promoting an overall pro-apoptotic effect on cancer cells.

MAPKs signaling pathway is an important upstream regulator of transcriptional factor activities and their signaling affects a wide variety of extracellular stimuli into intracellular events and thus control the activities of downstream transcription factors implicated in cancer development and progression [156]. GSE has been reported by many studies to enhance the activation of JNK and p38MAPK, through a pathway requiring intracellular calcium increase [103,157]. In turn, p38MAPK enhances apoptosis through Bcl-2 inactivation, caspase increase and mitochondria depolarization [158]. That effect has been related to ROS [159] and intracellular calcium increase [103], and it is generally thought to participate in enhancing the overall GSE-induced apoptotic action on cancer cells. Yet, opposite findings have been recorded in normal cells [160]. Moreover, GSE and several different polyphenols from both grape and tea have been showed to exert contradictory effects on ERK1/2 activation: meanwhile some studies reported epigallocatechin-3-gallate phosphorylation of ERK1/2 [161], we and others have observed a selective inhibition of ERK phosphorylation in colon and prostate cancer cells treated with GSE [103,147,162,163], or even EGCG [164,165]. Indeed, both down- and up-regulation of ERK activation in cancer cells have been reported occurring after treatment with GSE or isolated polyphenols [115]. Those contradictory results may be ascribed to differences in the cell culture, to dose-dependent dual effects, or to the prevalence of a specific single bioactive component, given that opposite effects on ERK activation have been documented by using different single bioflavonoids [166]. Therefore, data provided by experimental models need to be interpreted according to a systemic approach, i.e. by taking into consideration the dynamic interplay of several other observables [167].

In some way GSE and many dietary pholyphenols seem also to modulate the complex array of PKC iso-enzymes, leading to increased PKCα activation [168]. GSE may activate PKC, namely the PKCα and PCKδ isoforms, probably by increasing intracellular Calcium [169], and promoting PCKδ translocation into the nucleus, where PKC act as pro-apoptotic factor [170]. PKCα, together with PCKδ, could participate in inhibiting Akt phosphorylation and in triggering the extrinsic apoptotic cascade, especially in prostate cancer cells [171]. However, the interplay in between GSE and PKC dynamics is very poorly understood and deserves further investigation.

Several studies have indicated that elevated levels of inflammation modulators are functionally related to tumor promotion. Prostaglandins are produced in abundance by the metabolic conversion of arachidonic acid by COX-2, which has been known to be upregulated in a number of malignancies. Four transcription factors including nuclear factor kappa B (NF-kB), CCAAT/enhancer-binding protein (C/EBP), activator protein 1 (AP-1) and CRE-binding protein (CREB) have been identified to bind to the cis-acting elements required to promote COX-2 expression [172]. Among the aforementioned factors, NF-kB and AP1 play a relevant role in cancer development and progression [173]. The NF-kB proteins can be activated by a wide variety of stimuli that relieve NF-kB from the inhibition exerted by IkBα. NF-kB is indeed constrained in the cytosol by binding to IkBα. NF-kB activation requires necessarily that this association be disrupted. Almost all activators of NF-kB do so by phosphorylating IkBα when bound to NF-kB–Ikα kinases resulting in accelerated degradation NF-kB and nuclear translocation of free NF-kB [174]. In the nucleus, NF-kB targets different gene promoters, enhancing pro-survival pathways and even COX-2 genes expression. *In vitro* treatment of human epidermoid carcinoma A431 cells with GSE down-regulates the constitutive expression or basal level of NF-κB/p65 and IKKα and simultaneously inhibits the degradation of IκBα protein [175]. Indeed, many polyphenols as well as GSE have been proven to down-regulate NF-kB [136,178-180], and COX-2 expression [179,180]. As for EGCG extracted from tea [181], NF-kB down-regulation by GSE may also involve inhibition of Her-2/neu receptor tyrosine phosphorylation, an oncogene member of the EGFR family thought to play a relevant role during cancer development. To our best knowledge, among dietary flavonoids, only EGCG [182], Flavones [183,184] and mangiferin [185] (an apple procyanidin), share with GSE that meaningful, inhibitory property on NF-kB activation. Eventually, GSE has been reported to down-regulate the activator protein-1 (AP-1) levels in cancer cells [186], likely through different, synergistic biochemical pathways, as it was demonstrated by using isolated polyphenols [187]. AP-1 is very often portrayed as a general, nuclear decision maker that determines the final fate of the cell upon stimulation by extracellular signals, and its down-regulation has been claimed to participate in inhibiting anti-apoptotic and pro-survival pathways [188].

Additionally GSE and tea polyphenols have been demonstrated to modulate androgen [189] as well as estrogen signalling [190,191], involving a plethora of growth factor, as EFG/EGFR [192], PDGF [193], VEGF [194] and IGFBP-3 [195]. Overall, these effects may converge towards the aforementioned pathways, enhancing the anticancer activity displayed by GSE on cancer cells.

Extrinsic and intrinsic apoptotic pathway

The process of apoptosis is highly complex, and involves a cascade of molecular events, distributed along two main pathways: the extrinsic and the intrinsic or mitochondrial-derived pathway [196]. Both of them interact in some way and eventually converge into the same executioner pathway leading to activation of caspase-3 and PARP [197]. The extrinsic pathway encompasses interactions in between transmembrane death receptors, including DR4 and DR5, two members of the tumor necrosis factor (TNF) receptor gene super family. Cooperative participation of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and the Fas associated death domain (FADD), leads to the formation of the death-inducing signaling complex (DISC), which triggers the autocatalytic cleavage/activation of procaspase-8. Activation of caspase-8 ends-up eventually in activation of terminal caspases (caspase-3) and PARP. On the other hand, intrinsic pathway is activated by several different stimuli, leading to a dramatic decrease in transmembrane mitochondrial potential and consequently release of cytochrome c and pro-apoptotic effectors across the mitochondrial membrane. Further events largely depend on the balance between pro-apoptotic or anti-apoptotic proteins released from mitochondria. Anti-apoptotic effectors include Bcl-2, Bcl-xl; pro-apoptotic proteins are represented by Bik, Bim, Bak, Bad and Puma. Activation and stabilization of p53, reduced levels of MDM2 and PI3K and phosphorylated Akt, may synergistically shift the equilibrium towards pro-apoptotic proteins, eventually enhancing the system in moving to the next apoptotic steps. Increased levels of p53, in turn, may lead to augmented levels of Puma, Bak and p21, which translocate into mitochondria reinforcing membrane depolarization and further cytochrome c release and ROS formation [198].

Compelling data have been reported evidencing GSE and many of its bioflavonoids induce apoptosis in cancer cells through both the intrinsic as well as the extrinsic pathway by down-regulating anti-apoptotic proteins and up-regulating several pro-apoptotic fact eventually leading to activation of caspases 9 and 3 [116,132,137,139 1 9 9 - 201]. Those effects are highly dependent on inhibition of the PI3K/Akt/survivin pathway [202], and on the p38MAPK/JNK/ERK modulation [203], and have been recently confirmed by *in vivo* studies [204,205]. Moreover, that pro-apoptotic effect is highly specific, as normal cells are generally insensitive to GSE and other dietary polyphenols [137,205,206]. It is worth of noting that such effects occur irrespective of the p53 status of the cells. GSE was originally reported to induce apoptosis in a greater extent in p53-expressing cancer cells (through up-regulation of p53, Bak, p21, and Puma), than in p53-deficient samples [207]. Indeed, some GSE components specifically recognize p53 as a target and lead to p53 activation by binding and interacting to integrin αvβ3 [208]. Furthermore, a selective enhancing effect on p53 has been attributed to both ellagic acid [209] and EGCG [210], which seems to mandatory require p53 to exert its anti-apoptotic effects on cancer cells [211]. However, several studies found that the cytotoxic effect exhibited by the overall extract from grape seeds is actually independent of p53 status of the cancer cell lines [57,133,212]. That observation is highly significant, given the fact that one of the most common genetic defects found in cancers involves deletion/mutation of the TP53 gene, which encodes for the p53 protein [213].

The caspase-dependent pathway might not be the only apoptotic mechanism triggered by GSE (at least in colon cancer cells), bearing in mind that a slightly rise in cleaved PARP may be recorded before an increase in caspase activity could be observed. Indeed, we have shown that apoptosis inducing factor (AIF), known to induce apoptosis via a caspase-independent mechanism, increases early in GSE-treated samples and anticipate caspase-dependent apoptosis [137]. Those results have been further confirmed [109]. Furthermore, both caspase-dependent and caspase-independent apoptosis has been documented in prostate cancer cells after GSE treatment. Even in this case, addition of the ROS-inhibitor NAC prevents almost completely the grape-induced programmed cell death [214]. Involvement of AIF-mediated apoptosis in EGCG-treated cancer cells has also been documented [215,216]. Thus, GSE-induced apoptosis in several cancer cell lines can be considered a biphasic process, obtained through both caspase-dependent and caspase-independent pathways.

Cytoskeleton, ECM-Interactions and EMT-Transition

GSE anticancer actions are not restricted to cell growth inhibition and promotion of apoptosis. Some studies have linked the anti-cancer properties of dietary and tea polyphenols to the induced changes in cytoskeleton architecture and matrix metalloproteinases (MMPs) expression. Indeed, tea flavonoids down-regulate F-actin and 67 kDa-laminin receptor, thus inhibiting the myosin II regulatory light chain [217]. Those effects involve also polyphenols-binding to α2β1-integrin, followed by reorganization of the cytoskeleton, phosphorylation of focal adhesion kinases, and MMPs down-regulation [218].

Similarly, some preliminary reports suggest that cytoskeleton could be a target for GSE activity. Very interesting data have showed that GSE interact with some cytoskeletal proteins, favouring the cytosolic re-localization of β-catenin and down-regulating fascin expression [135]. Fascin is a highly conserved actin-bundling protein that localizes to microspikes and filopodia, participating in motility control [219]. Fascin has been found over-expressed in large numbers of metastatic cancers [229]. Fascin expression is significantly down-regulated by adding GSE to breast cancer cells culture, thus hindering the motility capability of treated cells, as evidenced by the migration assay [135]. Indeed, GSE-treated cancer cells exhibited less motility and invasiveness, a meaningful effect that should be ascribed, at least in part, to the inhibited activity of different MMPs. Conversely, we have observed [135] that GSE greatly inhibited MMP-2 and MMP-9 expression, as well as urokinase-type plasminogen activator (uPA), a key factor which mediates cellular invasion both directly by degrading members of the matrix proteins [221] and indirectly by modulating MMPs activation. A similar pattern has been observed by treating cancer cells with resveratrol [222], resveratrol analogues [223], or other dietary proto-anthocyanidins [224] and polyphenols [225-227]. It is interesting that effects on metalloproteinases, at least when referred to EGCG-treated cancer cells, have been deemed as a consequence of increased release of super oxygen radicals [228]. Additionally, it has been reported that GSE enhances the levels of epithelial (E-cadherin, cytokeratins and desmoglein-2) and reduces the levels of mesenchymal (vimentin, fibronectin, N-cadherin and Slug) biomarkers [144]. Overall those results suggest that GSE may efficiently counteract the epithelial-mesenchymal transition as well as the invasivity of cancer cells, by remodelling the cell-matrix interactions and stabilizing collagen architecture [229,230]. Those findings shed light on the mechanisms supporting the observed anti-metastatic property of GSE. Indeed, preliminary experimental investigations on animals showed that GSE significantly inhibits lung [136] and bone [231] metastasis from mouse breast cancers. Such preliminary results disclose a very new perspective in understanding the anticancer effects displayed by GSE.

Conclusion

Since ancient times, in various cultures and religions, there has been a strong belief that alcohol offers important health benefits. In recent years, the idea that regular, moderate alcohol consumption protects against cardiovascular disease and degenerative diseases has gained momentum.

A large number of studies have shown a significant inverse relationship between wine and/or grape consumption and mortality from all causes [232]. Specifically, a moderate wine consumption as well as regular intake of grape fruits seem to ensure an overall benefit in reducing the risk of dying from heart disease or cancer by approximately 40-60% [233,234]. During the last two decades, those data received a convincingly confirmation by a huge body of experimental investigations carried out *in vitro* as well *in vivo* models [235,236], demonstrating how GSE can hamper carcinogenesis and even counteract cancer development and progression, by inducing apoptosis and cell cycle arrest.

Is rather paradoxical that GSE promotes such effects by first enhancing intracellular ROS. ROS are thought to play a relevant role at the very beginning of the carcinogenic process, indeed. However, they behave really akin a double-edged sword, given that when their intracellular levels overwhelm the cellular antioxidant capacity, ROS increase ends up being detrimental to cells survival [237]. By taking into consideration how important ROS-induced apoptosis could be in improving current therapeutic anticancer strategies without adversely affecting normal cells [238], it can be concluded that GSE supplementation promises to be a reliable, new pharmacological opportunity that deserves much more thoughtfulness in both experimental and clinical studies. Indeed, GSE have shown that it is well tolerated and is considered safe as dietary supplement for human consumption, even at the highest doses and for long-lasting period of administration [239,240].

Both epidemiological and experimental studies currently support the beneficial effects of dietary pholyphenols and namely, of GSE. Several bioactive compounds have been identified and their activity has been documented, both *in vitro* and in vivo. However, the key question here is whether a purified component (whatsoever its effectiveness should be) has the same health benefit of the mixture from which it has been extracted. Indeed, it has long be recognized that GSE displays synergistic effects and additive interactions that potentiate the activity of individual components, thus suggesting that these compounds will exert their bioactivities only when harvested or delivered as natural mixtures from plant cell donors [241,242]. Likewise, although most experimental data have demonstrated the relevant anticancer role sustained by EGCG as the prevalent green tea constituent, the overall biological activity of green tea is thought to require the cooperative action of several components, rather than a single molecule [243,244]. Dietary phytochemicals are generally embedded into complex mixtures that often act in a synergistic fashion [245,246]: the isolated pure compound either loses its bioactivity, becomes unstable, or may not behave the same way as the compound in whole foods, as it has been demonstrated for a lot of phytochemicals, including the well-known anti-oxidant vitamin C [247,248]. In addition, diversity in molecular size, polarity, and solubility, may affect the bioavailability and distribution of each phytochemicals in different macromolecules, subcellular organelles, cells, and even tissues. Those considerations may explain the contradictory, even paradoxical results obtained in chemopreventive trials by using purified, single compounds [249,250]: no single molecule can replace the combination of natural phytochemicals in fruits, vegetables or overall grape seed extract in achieving the observed health benefits.

Thus, it is now "widely believed that the actions of the dietary supplements alone do not explain the observed health benefits of diets rich in fruits, vegetables, and whole grain, because, taken alone, individual molecules studied in clinical trials do not appear to have consistent preventive effects" [251].

Compelling data strongly suggest that grapes and grape-based products exert significant anticancer effects, as demonstrated by studies performed on cell culture and animal models. Grape polyphenols enhance ROS levels in cancer cells, leading then to a wide array of molecular and genetic changes, including phosphorylation of MAP-kinases, inhibition of PI3K-Akt and NF-kB pathways, down-regulation of cyclins and CDKs, and activation of both extrinsic and intrinsic apoptotic pathways. By that way, GSE selectively hinders cell proliferation and strongly enhances apoptosis. Despite the many challenges for dietary natural products caused by lack of standardization, composition variability and the limited reporting of adverse effects [252], such 'mixtures' have a great relevance as source of potential new pharmacological molecules and may represent an important opportunity for clinical research that should not be neglected. Considering the limited therapeutic options still available against several types of cancer, results herein reported indicate GSE could be thought as a new valuable treatment. Yet, clinical studies are urgently warranted in order to support this attractive hypothesis.

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