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***Chapter 20***

# GRAPE POLYPHENOLS AS POTENT INHIBITORS OF ANGIOGENESIS FOR THE CHEMOPREVENTION AND TREATMENT OF CANCER

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## ABSTRACT

Angiogenesis, the process of new blood vessels formation, comprise a highly regulated process which is pivotal for many physiological events such as embryogenesis, organ development, female reproductive cycle and wound healing. On the contrary, the unregulated angiogenesis is associated with the development of various states of diseases such as retinopathy, rheumatoid arthritis, psoriasis, atherosclerosis, haemangioma and cancer. In respect the cancer, it is now established that the angiogenesis is associated with the growth, progression and metastasis of solid malignant tumors beyond a certain size (>2 mm diameter), constituting an obligatory process for their adequate nutrients supply. Several studies have linked the over-expression of the main angiogenic factor VEGF (Vascular Endothelial Growth Factor) with a more aggressive behavior of the growing tumors. Thus, the development of specific antiangiogenic agents represents an attractive therapeutic approach for the chemoprevention and treatment of cancer, emerging the need for the development of novel (natural or synthetic) antiangiogenic molecules. In this context, the exploitation of grape (*Vitis vinifera*) origin polyphenols comprises an intriguing endeavor, since many of them have been found to display potent antiangiogenic properties, mainly through the VEGF inhibition. This review aspires to account and present –for the first time– the literature reports on research concerning the angiogenesis inhibition derived anticancer properties of grapes, and grape derived polyphenols (flavonoids, phenolic acids, stilbenes, etc.) and products (wines, juices, extracts). The topic is presented as a literature overview focusing on the results published during the new millennium and targeting the chemoprevention and treatment of cancer.

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## INTRODUCTION

Angiogenesis, the process of new blood vessels formation, is pivotal for many physiological events including embryogenesis, organ development, the female reproductive cycle and wound healing [1,2]. Under these conditions, angiogenesis is a highly regulated process, i.e. turned on for brief periods and then completely inhibited. On the contrary, unregulated angiogenesis has been associated with the development of diverse diseases states, including retinopathy, rheumatoid arthritis, psoriasis, atherosclerosis, haemangioma and cancer [3-5]. Angiogenesis constitutes a complex process controlled by some 20 endogenous activators and more than 28 inhibitors. In healthy adult bodies angiogenesis is controlled by an equilibrium of the released −from healthy cells− angiogenic factors, while the excessive or insufficient angiogenesis is connected with the pathology. Thus, inhibitors and stimulators of angiogenesis may provide powerful new weapons in the armamentarium against cancer and other illnesses such as heart and eye disease.

The role of angiogenesis and contribution on tumor progression was firstly recognized by Judah Folkman in early 70's, through the revolutionary for that time concept (published in The New England Journal of Medicine) that tumors are unable to grow beyond a certain size, unless they develop a dedicated blood supply [1]. In particular, "successful" tumors promote the growth of new blood vessels which are essential for their nutrients-oxygen supply and waste control, in order be transformed from small clusters of mutated cells to large, malignant ones. A similar mechanism also contributes for the cancer cells spread during the metastatic processes. Thus, the event of angiogenesis is essential for the growth and progression of solid tumors beyond 2 mm diameter size (imposed by simple diffusion for their nutrients supply), constituting the foundation for their inhibition therapy. The latter is based on Folkman's elegant principle stating that "instead of waging a battle with a tumor using toxic chemicals and radiation, one could starve it by shutting down its blood supply". Today, almost 40,000 papers have been published on this subject [6], at least 50 angiogenesis inhibitors are in clinical trials and more than 1,000 laboratories (in universities and industry) conduct angiogenesis research. It is estimated that eventually the inhibition of angiogenesis will become the 4th modality in treating cancer (after surgery, radiation and chemotherapy) and someday the angiogenesis inhibitors will be routinely used together (or in combination) with conventional anticancer therapies such as chemotherapy, radiotherapy, immunotherapy and gene or vaccine therapy.

Despite the very promising results obtained for the antiangiogenic treatment in animal models and the safety features of antiangiogenics developed to date, their application in humans is not yet as effective as expected, emerging the need for the development of new (synthetic or natural) highly active angiogenic inhibitors. In this context, the exploitation of plant derived products comprises a promising choice, since nature has inherently served as a reservoir of potent bioactive molecules and the starting point for the development of many pharmaceuticals. In this respect, among the plethora of bioactivities assessed for plant phytochemicals, many extracts (and natural compounds) isolated from various plant families such as *Berberidaceae*, *Rosaceae*, *Zingiberaceae*, *Cephalotaxaceae*, *Taxaceae*, etc., have been found to display significant antiangiogenic properties [7,8]. Among them, the exploitation of grapes (*Vitis vinifera*, *Vitaceae* family) presents an intriguing option, since grape polyphenols and extracts have been found to display significant antiangiogenic

properties [9-11], mainly through the inhibition of the main angiogenic factor, the Vascular Endothelial Growth Factor (VEGF).

Grapevine constitutes one of the most valuable horticultural crops of the world, cultivated in more than ~8 million *ha*. Most of the produced grapes are processed for the production of wine and the remaining are consumed as fresh table grapes or dried into raisins, distilled into spirits and processed into nonalcoholic juices (http://faostat.fao.org/). Currently, wine and gapes receive a noticeable research and public attention, mainly as a consequence of numerous epidemiological studies which have linked their regular consumption with the decreased risk of developing cardiovascular events, such as myocardial infarction, Coronary Heart Disease (CHD), stroke and cardiac death [11-14]. These health beneficial effects are associated with the ability of grape polyphenols to prevent the lipid peroxidation, increase the high-density lipoprotein cholesterol (HDL-C) levels, inhibit the platelet aggregation [15, 16], retarding the progression of early atherosclerotic lesions to advanced plaques, which are prone to rupture with superimposed thrombosis [17,18]. Some of these events are also attributed to the ability of grape polyphenols to control the angiogenesis (formation of new blood vessels). However, during the last years, most of the antiangiogenic properties of grapes polyphenols were linked with their cancer chemopreventive and treatment abilities [19].

Main objective of this work is to present −for first time− an in depth overview of the angiogenesis inhibition derived anticancer properties of grape polyphenols (flavonoids, phenolic acids, stilbenes, etc.) and grape derived polyphenolic extracts. Grape polyphenols constitute a diverse group of secondary metabolites which are present in grapes, wine and vinification byproducts. Their content is greatly influenced by the grape variety, handling and maturation stage as well as the technological practice to which grapes are exposed [20, 21].

## WINE

Epidemiological studies have established that the regular consumption of red wine is associated with a reduced risk of developing coronary heart disease and tumor progression. Since the development of tumors is advanced by the formation of new blood vessels that provide oxygen and nutrients to the neighboring cells, several studies have been directed towards the assessment of the antiangiogenic properties of wines [9]. Literature abounds with results revealing the *in vitro* experimental potency of wines to inhibit various key events of the angiogenic process, such as the proliferation and migration of endothelial and Vascular Smooth Muscles Cells (VSMCs) as well as the expression of the two major proangiogenic factors, namely the Vascular Endothelial Growth Factor (VEGF) and the Matrix MetalloProteinase-2 (MMP-2).

Red wine polyphenols (RWPs) have been recognized as strong inhibitors of the growth factor which is induced by the VEGF expression in VSMCs, at concentrations that are likely to be achieved in blood after the moderate consumption of red wine [22]. It is also established that the major enzymatic source of the reactive oxygen species (ROS) in VSMCs as response to growth factors, is the oxidase of the nicotinamide adenine dinucleotide phosphate (NADPH), since the VSMCs exposure to thrombin or $PDGF_{AB}$ causes the generation of substantial amounts of ROS via the activation of a p22phox-containing NADPH oxidase [23].

Thus, the antiangiogenic activity of RWPs may be rationalized considering their well established ability to display strong antioxidant properties, acting either as direct ROS scavengers [24] or as potent inhibitors of the NADPH oxidase expression and the xanthine oxidase activity [25].

The MMP-2 is expressed abundantly in atherosclerotic and restenotic lesions, playing a key role in the basement membrane degradation, thereby promoting the migration of ECs and VSMCs. Recent studies have indicated that RWPs strongly inhibit the VSMC invasion, induced by growth factors such as thrombin and PDGFBB [26]. Their inhibitory effect is closely associated with the concentration-dependent inhibition of thrombin-induced MMP-2 activation [27] and the RWPs ability to inhibit the MT1-MMP activity, when added directly to the enzymatic assay. These findings suggest that the polyphenolic extracts of red wines prevent the MMP-2 activation through the direct inhibition of the activity of the membrane-bound MT1-MMP. It must be noted however, that the identity of most active polyphenols of red wines is still ambiguous, though relative studies have indicated that catechin display the capability to prevent the MT1-MMP dependent activation of MMP-2 in cancer cells [28] and *trans*-resveratrol is a strong inhibitor of the MMP-2 expression and activity in different types of cells [29].

The *in vivo* evaluation of the angiogenesis inhibition potency of the red wine polyphenolic extracts or pure polyphenols are scarce, and mainly concern the chicken embryo ChorioAllantoic Membrane (CAM) assay. The latter accounts the reduction (in number and length) of the small new blood vessels formation after a 48-h incubation period [9]. Among the red wine polyphenols, the molecule of *trans*-resveratrol has been assessed to exhibit significant inhibitory activity on the corneal neovascularization induced by VEGF and bFGF in mice [30] and the *in vivo* tumor growth and tumor-induced neovascularization [31].

An interesting result concerning the peculiar dual behavior of wine polyphenols which display protective role against the cardiac and cerebral ischemia and angiogenesis inhibition activity, was revealed by Baron-Menguy et al. [32] during an *in vivo* study of the RWPs dose-dependent effect on angiogenesis model triggered by ischemia. More specifically, they treated rats with low (0.2 mg/ kg/day) and high (20 mg/kg/day) doses of RWPs, which were submitted to the femoral artery ligature on their left leg. Two weeks after the ligature, they observed that high doses ( about 7 glasses of red wine) reduced the arterial, arteriolar, capillary densities and the blood flow. They also observed an inhibition of the PI3 kinase-Akt-endothelial NO synthase (eNOS) pathway and a sharp decrease in VEGF expression and MMP activation. On the contrary, low doses (0.1 glass of red wine) increased the blood flow, the microvascular density and the left/right (L/R) leg ratio to control level [32]. This angiogenic effect is attributed to the PI3 kinase-Akt-eNOS pathway over-expression and the increase of the VEGF production, while the the MMP activation was not affected. Thus, low and high doses of RWPs display *in vivo* pro (or anti) antiangiogenic properties respectively on postischemic neovascularization. This unique dual effect of wine polyphenols offers important perspectives for the prevention of ischemic diseases (low dose) or cancer growth (high dose), since they were both determined as orally active, with no potentially serious adverse effects and high degree tolerability. Thus, they provide a good safety profile connected with low production cost.

The molecular mechanism associated with the *in vivo* antiangiogenic activities of red wine extracts (and polyphenols) is unclear, though is well estimated that relies on their ability to inhibit the following key events of the angiogenic process; proliferation and migration of

ECs and VSMCs, expression of VEGF and MMP-2 activation. It must be noted however, that a very important element to understand the biological activities of natural polyphenols is related to their bioavailability. Lack of this issue understanding may lead to excessive claims in respect to their *in vivo* biological activities. To date, the absorption of only very few red wine polyphenols has been studied, while the absorption of even fewer compounds has been evaluated within a wine matrix. Among them the absorption ability of red wines' flavanols, flavonols, anthocyanins and nonflavonoid stilbenes of red wine is well established [33] along with their inhibitory effect on proangiogenic responses, which started at concentrations as low as 3 μg/mL [22,34]. Although their concentration in blood after the intake of red wine remains unknown, a previous study has showed that the intake by healthy volunteers of 100 mL red wine increases the polyphenolic monomers concerntration in their plasma by 2.5 μg/mL (determined as gallic acid equivalents) [33]. In addition, the minimum concentration of (−)-epigallocatechin gallate (EGCG) necessary to initiatey significant anti-angiogenic effects in a mouse corneal model is in the range of 0.1–0.3 μM [9], indicating that the inhibitory effect of wine polyphenols on proangiogenic responses is likely to be reached in blood after moderate consumption of red wine.

## GRAPES

Grape skins are considered as a valuable source of numerous phytonutrients, including the intensively studied molecule of *trans*-resveratrol. Many studies have established that the consumption of grape derived products is beneficial for the prevention of cardiovascular diseases, Alzheimer's disease, urinary bladder dysfunction and various types of cancers [35]. The latter has recently attracted a significant attention due to many research endeavors highlighting the role of grapes and their extracts in the growth inhibition of various human and mouse tumor cells [36]. The pharmacological effects of grapes were recently reviewed by Hosseinzadeh [37] underlying the anticarcinogenic and antiangiogenic properties of grapes derived extracts and compounds. Barthomeuf et al. investigated the biological properties of red grape skin polyphenolic extracts (25 μg/mL), showing that they prevent and inhibit angiogenesis in the Matrigel model, decrease the basal motility of endothelial and cancer cells, reverse the chemotactic effect of sphingosine-1-phosphate (S1P) and VEGF on bovine aortic endothelial cells (BAECs) as well as the chemotactic effect of conditioned medium on human HT-1080 fibrosarcoma, human U-87 glioblastoma, and human DAOY medullo-blastoma cells [38]. The inhibition of VEGF and S1P mediated chemotaxis by the grape skin extracts has been associated with the down-regulation of ERK and p38/MAPK phosphorylation and a decreased in acute PAF synthesis. Notably, as do extracellular inhibitors of PAF receptor, grape skin extracts prevent the S1P induced PAF synthesis and the resulting activation of the S1P/endothelial differentiation gene-1 cascade. Given the key role of VEGF and S1P in inflammation, angiogenesis and tumor invasion, it is estimated that the grape skin extracts contribute to the prevention (or delay) of the development of diseases associated with angiogenesis dysregulation, including cancer. On the other hand, the dual inhibition of S1P and VEGF mediates the endothelial cells migration along with the serum-stimulated migration of the U-87 cells, indicating the grape skin polyphenolic extracts usefulness against the highly invasive human glioblastoma.

In another study Walter at al. showed that the grape-derived polyphenols display attractive anticancer properties, supporting their role as potential chemopreventive agents against cancer [39]. They also determined *in vivo* the antiangiogenic, antiproliferative and proapoptotic effects of the grape-derived polyphenols, which are associated with the effective inhibition of colon carcinoma tumor growth in mice. Finally, they showed that the red grapes polyphenols prevent effectively the formation of preneoplastic lesions, induced by a carcinogen at the colon mucosa in rats. Since a key hypothesis dictates that the tumor growth depends closely on the new blood vessels formation (controlled by the major proangiogenic factors VEGF and MMP-2), they performed several experiments to delineate whether the red wine polyphenols are capable to retard the tumor growth by preventing its vascularization. The respective results indicated that polyphenols reduce by 40% the blood vessels volume in tumors. In addition, the immunohistochemical staining with CD31 (endothelial cell marker) allowed the determination of blood vessels in tumors, evidencing a 53% reduction of the microvessels density in tumors. The investigation of grapes polyphenols effects on the expression of the major proangiogenic factors in tumors revealed that they act through mechanisms involving the VEGF, MMP-2 and COX-2. Concomitantly it was shown that the inhibition of tumor angiogenesis by red grape polypehnols proceeds via the expression of the major proangiogenic factors. These findings were reinforced by various experiments concerning the inhibition of tumor cell proliferation and metastasis of various lung cancers [39]. Finally, several studies have revealed the ability of red wine polyphenols to inhibit the colon carcinogenesis induced by azoxymethane in rats [40] and the capacity of the molecule of *trans*-resveratrol to reduce the growth of glioma in rats [41].

## GRAPE SEEDS

Grape seeds contain the 60-70% of grapes polyphenols. They constitute vinification byproducts which are being used as raw material for the preparation of extracts (grape seed extracts, GSEs) or seed oil. The commercial preparations of GSEs are of great economic interest, since they are marketed as dietary supplements due to their powerful protective properties against free radicals and oxidative stress. GSEs have also been linked with the cancer prevention and therapy, since they have been determined as potent inhibitors of the growth of numerous *in vitro* cancer cells [42] and various *in vivo* tumors in mice [43,44]. Research towards the elucidation of their anticancer activities mechanism revealed their ability to inhibit the endothelial cell proliferation and tube formation on Matrigel [45] and reduce the vessels density in human prostate tumors [46]. Studies of Wen et al. concerning the GSEs activity on the VEGF receptor and angiogenesis inhibition [47] showed that GSEs inhibit directly the kinase activity of purified VEGF receptor 2, a novel activity which has not been characterized before. In addition, they showed that in endothelial cells the GSEs act as potent inhibitors of the VEGF receptor/mitogen activated protein kinase–mediated signaling pathway. As a consequence, GSEs display the ability to inhibit the VEGF-induced endothelial cell proliferation and migration, and sprout their formation from aorta ring. Experiments *in vivo* also revealed that the GSEs are potent inhibitors of the tumor growth and tumor angiogenesis of MDA-MB-231 breast cancer cells in mice. Consistent with the corresponding *in vitro* data, the treatment of tumor-bearing mice with GSEs reduced the density of the blood

vessels and the phosphorylation of mitogen-activated protein kinase. On the other hand, the polyphenols depletion with polyvinylpyrrolidone resulted in the abolishment of the GSEs antiangiogenic activities, suggesting that a water-soluble polyphenolic fraction of GSEs is responsible for their antiangiogenic activity [47].

The antiangiogenic efficacy of GSEs was also studied *in vitro* by Agarwal et al., who used human umbilical vein endothelial cells (HUVEC) cultures in order to evaluate their proliferation, survival, MMPs secretion and capillary tube formation potentials [45]. In this respect, they determined that the GSEs are capable to inhibit significantly the cell growth (<91%) and cell viability (<64%) of HUVEC. In addition, they showed that the GSEs strongly inhibit the DNA synthesis (<76%) and induce apoptotic cell death (<42.8% versus 2.6% of control), both in HUVEC. Other experiments showed that GSEs treatment decreased the secreted levels of MMP-2 from HUVEC and inhibited the capillary tube formation on Matrigel by the endothelial cells in a dose dependent manner. The aforementioned findings suggest that GSEs possess an anti-angiogenic potential, which is associated with its antiproliferative, proapoptotic and inhibition of MMP-2 secretion in endothelial cells.

A new insight into the mechanism of anticancer activity of GSEs, which reveals a novel molecular mechanism that underlies their antiangiogenic activity, was reported by Wen et al. [48]. They evaluated the GSEs inhibitory effect on the VEGF expression and the respective mechanism of action, showing that GSEs inhibit the VEGF messenger RNA (mRNA) and protein expression in U251 human glioma cells and MDA-MB-231 human breast cancer cells. Furthermore, GSEs inhibit the transcriptional activation of the VEGF gene through the protein reduction and not the mRNA expression of hypoxia inducible factor (HIF)-1α. The inhibitory effect of GSEs on HIF-1α expression was mainly expressed through the inhibition of HIF-1α protein synthesis rather than the promotion of the protein degradation. Consistent with this result was the GSEs-suppressed phosphorylation of several important components involved in the HIF-1α protein synthesis, such as Akt, S6 kinase and S6 protein.

Furthermore, in the MDA-MB-231 tumor, they determined that the treatment with GSEs inhibits the VEGF and HIF-1α expression and the phosphorylation of S6 kinase, without altering the subcellular localization of HIF-1α, which is correlated with a reduced vessel density and tumor size.

Several *in vivo* studies delineated the relationship between the GSEs consumption and the growth inhibition of advanced human prostate cancer, elucidating simultaneously the role of the associated molecular events (antiproliferative, apoptotic and antiangiogenic) [49,50]. In this respect, athymic nude mice were implanted with hormone-refractory human prostate carcinoma DU145 cells and fed with 100 and 200 mg/kg/day (5 days/week) doses of GSEs for 7 weeks. At the end of the experiment, the tumors were immunohistochemically analyzed for cell proliferation, apoptosis and angiogenesis. The data obtained showed that the GSEs feeding strongly inhibited the tumor growth, producing a 59–73% inhibition of tumor volume and 37–47% decrease of tumor weight at the end of the experiment. The immunohistochemical analysis of tumors showed that the GSEs decreased the proliferation index by 51–66% and increased by 3–4 folds the apoptotic index. The CD31 staining of endothelial cells showed a decrease in the intratumoral microvasculature for the GSE-fed group of mice. Control tumors showed ~64 CD31 positive cells/400 per field, as compared with ~23.2 and ~15.7 CD31 positive cells observed for 100 and 200 mg/kg doses respectively, for GSE-treated tumors. Finally, it was determined that the GSEs consumption strongly inhibits (47–70%) the VEGF secretion in the conditioned medium by DU145 cells.

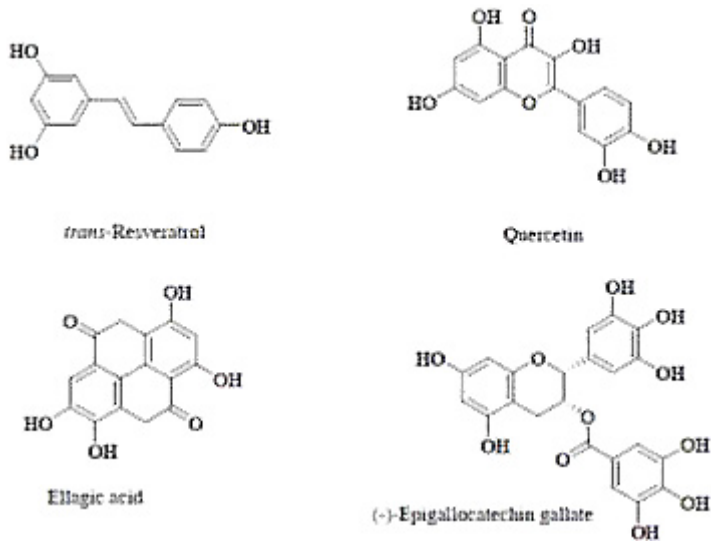


Figure 1. Chemical structures of selected grape polyphenols.

## BIOACTIVE POLYPHENOLS

Grapevines (*Vitis vinifera*) are known to contain a broad variety of polyphenols (Figure 1) with pronounced bioactivities (eg. antioxidant, cardioprotective, anticancer, estrogenic, antiangiogenic, etc.) [35,37]. These natural molecules are present in grape skins, seeds, stems, pomace as well as the produced wines and juices as pure compounds, as polymers (dimmers, trimers, etc.) or as their glucosides. Structurally, they are classified in accordance to their chemical backbone in classes such as flavonoids, phenolic acids, stilbenes, and so on. Below are presented the polyphenols that have been reported to display well established antiangiogenic properties, associated with their role in the chemoprevention and/or treatment of various types of cancer tumors.

### A) *Trans*-Resveratrol

*Trans*-resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring phytoalexin produced by a wide variety of plants in response to stress, injury, ultraviolet (UV) irradiation and fungal (e.g. *Botrytis cinerea*) infection. There are several plants, including grapevine, which upon attack by pathogens biosynthesize resveratrol from the precursor molecules malonyl-coenzyme A (CoA) and *p*-coumaroyl-CoA in the presence of stilbene synthase. Though resveratrol was firstly isolated in 1940 as a constituent of the roots of white hellebore, red grapes (and wine) constitute its most known and investigated source. It must be noted however, that except the *Vitis* spp. (grapevines, leaves, and berryskins), resveratrol is found in a wide variety of plants, including Japanese knotweed (*Polygonum cuspidatum*), peanut, *Vaccinum* spp. (including blueberry, bilberry, and cranberry), *Reynoutria japonica*, Scots pine, *Morus* spp. (including mulberry), lilies (*Veratrum* spp.), legumes (*Cassia* spp., *Pterolobium hexapetallum*), *Rheum* spp. (including rhubarb), eucalyptus, spruce (*Picea* spp.),

pine (*Pinus* spp.), grasses (Poaceae including *Festuca, Hordeum, Poa, Stipa,* and *Lolium* spp.), *Trifolium* spp., *Nothofagus* spp., *Artocarpus* spp., *Gnetum* spp., *Pleuropterus ciliinervis*, *Bauhinia racemosa*, *Paeonia lactiflora*, *Scilla nervosa* and *Tetrastigma hypoglaucum*.

The fresh grape skins contain 50-100 mg resveratrol per gram, while its concentration in wine ranges from 0.2 to 7.7 mg/L. The epidemiological finding that connects the moderate consumption of red wine with the reduced risk of developing cardiovascular diseases, widely known as "French paradox", is attributed −for a variety of reasons− mainly to their resveratrol content [51-53]. These effects are connected with the ability of this molecule to suppress the lipid peroxidation and eicosanoid synthesis, inhibit the platelet aggregation and display significant antioxidant, antiinflammatory and vasorelaxant properties [54]. Besides its cardioprotective ability, resveratrol is also known to display potent antiaging effects [55], antiviral activities against HIV-1 [56] and the herpes simplex virus [57], and antibacterial properties [58], including the growth inhibition of different strains of *Helicobacter pylori* [59]. Resveratrol is also known to enhance synergistically the anti-HIV-1 activity of the nucleoside analogues zidovudine (AZT), zalcitabine (ddC), and didanosine (ddI) [56]. Its health beneficial effects are also supported by data on humans revealing that resveratrol is pharmacologically quite safe [60].

During the last decade numerous research results have suggested that, besides the aforementioned health beneficial properties, resveratrol displays potent anticancer (chemopreventive and therapeutic) activities [60]. The latter are associated with resveratrol's ability to suppress the proliferation of a wide variety of tumor cells, including the lymphoid and myeloid cancers, the multiple myeloma, the breast, prostate, stomach, colon, pancreas and thyroid cancers, the melanoma, the head and neck squamous cell carcinoma, the ovarian carcinoma, and the cervical carcinoma [61]. These tumor growth-inhibitory effects of resveratrol are mediated through numerous pathways, including angiogenesis. The latter is a widely recognized essential process for the growth and progression of solid tumors, since provides the necessary means for nutrients supply of tumors. Thus, the detailed determination of the antiangiogenic properties of resveratrol is crucial step for the evaluation its anticancer potentials. Various studies have indicated that this molecule display the ability to act as potent inhibitor of angiogenesis by suppressing the FGF-2 and VEGF-induced *in vivo* neovascularization [30]. In addition, resveratrol inhibits directly the *in vitro* bovine aorta endothelial cell (BAEC) proliferation, migration and tube formation [62].

The event of angiogenesis involves a complex sequence of events, since during the angiogenic stimulation; the vascular endothelial cells increase their expression and secretion of matrix metalloproteinases (MMPs) to break down the extracellular and tissue matrix. Furthermore, increase the endothelial cell motility and undergo cell proliferation to provide the necessary number of cells for the growing vessels [63]. In this respect, a study was published in 2003, revealing that the exposure of human umbilical endothelial cells (HUVECs) to 1 to 2.5 μM resveratrol blocks significantly the VEGF-induced angiogenesis (migration and tube formation) [64]. In addition, 1 or 2.5 μM doses of resveratrol abrogate effectively the VEGF-mediated tyrosine phosphorylation of the vascular endothelial (VE)-cadherin and its complex partner β-catenin. Further *in vitro* and *ex vivo* experiments indicated that resveratrol has the ability to inhibit directly the growth of human umbilical vein endothelial cells and decrease the gelatinolytic activities of the MMP-2 [65]. The treatment with resveratrol was found to inhibit the tube formation (after plating endothelial cells on

Matrigel) and the endothelial cell attachment to the basement membrane components fibronectin and laminin, while resveratrol is a potent inhibitor of angiogenesis in a rat aorta matrix culture model [65].

Additional *in vitro* experiments showed that resveratrol and quercetin (another grape polyphenol) inhibit the growth of BAEC in a concentration-dependent manner (6–100 μM). In particular, the BAEC migration was obviously inhibited by resveratrol and weakly by quercetin [62]. Similar results were also obtained for the lengths of the tubes, which were affected by resveratrol in the same ranges of concentration, while quercetin required doses above 100 μM to inhibit the tube formation. Resveratrol was also found to enhance the *in vitro* and *in vivo* myocardial angiogenesis in VEGF induction experiments regulated by thioredoxin-1 (Trx-1) and heme oxygenase-1 (HO-1) [66]. The exposure of human coronary arteriolar endothelial cells to resveratrol or Trx-1 on Matrigel provoked the accelerated tubular morphogenesis with the induction of HO-1 and VEGF expression. This angiogenic response was repressed by tin-protoporphyrin IX (SnPP), a HO-1 inhibitor, along with the down-regulation of the VEGF expression. On the other hand, rat neonatal cardiomyocytes treated with resveratrol expressed significantly the VEGF, Trx-1 and HO-1. Pronounced pro-angiogenic effects were also obtained when rats were administered orally with resveratrol (1 mg/kg per day) for 14 days. The abovementioned findings suggest that resveratrol mediates the neovascularization and cardioprotection through the Trx-1–HO-1–VEGF pathway [66]. Another study on resveratrol's effects on the vasculogenesis of the yolk-sac membranes showed that high concentrations of resveratrol (43.8–438 μM/implant) significantly inhibit the early vessel formation, decreasing the percentage of vitelline vessels for 3.5-day embryos by 50% as compared to the control [67].

In respect the tumor models tested, it must be noted that the treatment of human leukemia U937 cells with different concentrations of resveratrol (12.5–200 μM/L) for different time lengths (12–48 h), was indicative of its ability to inhibit the proliferation of U937 leukemia cells through the down-regulation of VEGF secretion and induction of apoptosis [68]. Similar results were obtained in a series of experiments concerning the role of resveratrol in the suppression of angiogenesis, the tumor growth (murine fibrosarcoma in mice) and wound healing [30]. Resveratrol was also found to induce the transcription via both estrogen receptors (ERα and ERβ). In this respect, a significantly lower tumor growth, decreased angiogenesis and increased apoptotic index was observed for ERα(−) and ERβ(+) MDA-MB-231 tumors in resveratrol-treated nude mice [69]. Finally, during *in vitro* experiments a significant increase in apoptosis and reduction in the extracellular levels of VEGF were observed for the resveratrol-treated MDA-MB-231 cells. These results suggest that the molecule of resveratrol possesses a great potential for application as a chemotherapeutic agent in breast cancers.

In a different line of experiments, resveratrol was assessed as potent inhibitor of the multiple myeloma angiogenesis through the regulation of the expression and secretion of VEGF, bFGF, MMP-2 and MMP-9. Multiple myeloma is a B-cell malignancy characterized by the clonal expansion of malignant plasma cells in the bone marrow. More specifically, RPMI 8226 cells were co-cultured with human umbilical vein endothelial cells (HUVECs) to determine the myeloma cells effects on angiogenesis. The RPMI 8226 cells were treated with various concentrations of resveratrol (6.25–50.00 μM/L) for different time intervals (12–72 h) [70]. Results indicated that the cell proliferation, migration and differentiation of HUVECs

were markedly increased by their co-culture with RPMI 8226 cells. In addition, resveratrol inhibited the proliferation, migration and tube formation of HUVECs co-cultured with myeloma cells in a dose dependent manner. Treatment of RPMI 8226 cells with resveratrol caused a decrease in MMP-2 and MMP-9 activity, while resveratrol inhibited the VEGF and bFGF protein expression in a dose and time dependent manner. Furthermore, the decreased levels of VEGF, bFGF, MMP-2 and MMP-9 mRNA from cells treated with various concentrations of resveratrol confirmed its antiangiogenic activities at the level of gene expression. These results reveal that resveratrol represents a potent candidate for the treatment of multiple myeloma [70].

In an attempt to improve the bioavailability of resveratrol, many scientific projects were initiated towards the development and evaluation of its structural analogues [60]. These endeavors include the screening of antiangiogenic and anticancer activities of several naturally occurring stilbene glucosides, such as the molecule of resveratrol 3-*O*-D-glucoside (piceid). The inhibitory effects of the latter on the differentiation of HUVECs to form a capillary network were assessed, indicating that this molecule is a potent inhibitor of HUVECs capillary-like tube networks (angiogenesis) formation at concentrations ranging from100 to 1000 μM. In addition, the evaluation of piceid as inhibitor of lung tumor growth and metastasis in mice that bear the highly metastastic Lewis lung carcinoma (LLC), showed that its oral administration inhibits the tumor growth in the right hind paw and lung metastasis [71].

## B) Quercetin

Quercetin, the aglycone of rutin, is the most common flavonoid in nature, present in large quantitites in grapes, red wine and other food products. Besides its numerous biological activities, including antioxidant and antiinflammatory, there are several literature reports on its potent antiangiogenic and anticancer properties. More specifically, the activity of quercetin on choroidal and retinal *in vitro* angiogenesis was evaluated on rhesus choroids-retina endothelial cell line (RF/6A) in different concentrations (0 to 100 μM) [72]. The respective results are indicative of its ability to inhibit the endothelial cell proliferation in a dose-dependent fashion, accounting to 10.1%, 42.6% and 65.2% inhibition upon treatment with 10, 50 and 100 μM, respectively. Similar inhibition was observed for RA/6A cells, whilein another study quercetin was assessed as potent inhibitor (in a dose-dependent manner) of several important steps of the angiogenesis, including the proliferation, migration and tube formation of human microvascular dermal endothelial cells [73]. Additionally, the effect of quercetin on endothelial cell proliferation was confirmed using human umbilical vein endothelial cells, while the CAM assay revealed the *in vivo* antiangiogenic properties of quercetin.

Quercetin and its main circulating conjugates in humans [quercetin-3'-sulphate (Q3'S) and quercetin-3-glucuronide (Q3G)] were assayed for their *in vivo* potency to inhibit angiogenesis induced by VEGF [74]. Results herein indicated that quercetin and Q3G inhibit the VEGF-induced endothelial cell functions and angiogenesis, while the molecule of Q3'S promotes the endothelial cell proliferation and angiogenesis. The inhibitory effect elicited by Q3G was linked to the inhibition of the extracellular signal-regulated kinases 1/2 (ERK1/2) phosphorylation elicited by VEGF. The endothelial cells activation by Q3'S was attributed to

the capability of the molecule to stimulate the VEGF receptor-2 and activate the downstream signaling of phosphatidylinositol-3 kinase/Akt and nitric oxide synthase pathways, which are ultimately responsible for the ERK1/2 phosphorylation. These data indicate that the effects of circulating quercetin conjugates on angiogenesis are different and depend directly on the nature of the conjugate [74].

In a different line of experiments, quercetin was administrated for 28 weeks in seventy-nine female Sprague-Dawly rats with 7,12-dimethylbenzanthracene (DMBA)-induced animal mammary carcinoma [75]. Then, their breast tissues samples were examined through histopathological observation and microvessel density (MVD) estimation using light microscopy, while the expression of basic fibroblast growth factor (bFGF), VEGF and the H-ras protein were determined with immunohistochemical staining. Results indicated that quercetin is a potent reducer of the DMBA-induced mammary carcinoma and tumor growth, acting as inhibitor of the expression of angiogenesis-related growth factors such as VEGF and bFGF.

The investigation of the molecular mechanism involved in the quercetin-mediated amelioration of colonic mucosal injury revealed that proceeds through the VEGF up-regulation of an ulcer healing factor, not only in colon epithelial cell lines but also in the inflamed colonic tissue [76]. In addition, the VEGF derived promotion of tube formation from the quercetin-treated colon epithelial cells was also observed. This induction of VEGF depended directly on the quercetin-mediated hypoxia inducible factor-1 (HIF-1) activation. More specifically, quercetin delayed the HIF-1α protein disappearance, which occurred by inhibiting HIF-Prolyl hydroxylase (HPH), the key enzyme for HIF-1α hydroxylation and subsequent von Hippel Lindau-dependent HIF-1α degradation. These data suggest that the clinical effects of quercetin are attributed to the activation of an angiogenic pathway involving both HIF-1 and VEGF.

## C) Other Molecules

Ellagic acid (4,4',5,5',6,6'-hexahydroxydiphenic acid 2,6,2',6'-dilactone) is a naturally occurring dimeric derivative of gallic acid that is abundantly found in woody parts and berries of grapes [77]. Its bioactivity has been connected with various important health beneficial properties, including radical scavenging, chemopreventive and antiviral activities [78]. The cytotoxic and anti-proliferative activities of ellagic acid were evaluated in respect to its antiangiogenic properties, indicating that 1–100 μM/L doses of this molecules inhibits the HUVEC tube formation and proliferation on a reconstituted extracellular matrix. In addition, ellagic acid was determined to possess strong anti-proliferative activities against colon, breast and prostatic cancer cell lines [79]. Among cells tested the most sensitive were the Caco-2, while the most resistant were the breast cancer cells. Ellagic acid was also induced the death of cancer cells by apoptosis which was accompanied by a decreased of pro-MMP-2, pro-MMP-9 and VEGF levels. These results are indicative of the molecule's ability to express selective cytotoxic and anti-proliferative activities and induce apoptosis in Caco-2, MCF-7, Hs 578T, and DU 145 cancer cells without any toxic effect on normal human lung fibroblast cells viability. The mechanism of apoptosis induction in ellagic acid–treated cancer cells is associated with a decreased ATP production, which is crucial for the viability of cancer cells.

(−)−Epigallocatechin gallate (EGCG), the most abundant catechin derivative of green tea, which is also present in grapevines, is well known as a potent suppressor of the cell proliferation and inducer of apoptosis in colon cancer cells, acting as activation inhibitor of various types of receptor tyrosine kinases (RTKs). EGCG is also a potent inhibitor of VEGEF-2, which is associated with the *in vitro* suppression of angiogenesis [80].This molecule also suppresses the growth of human liver cancer cells by inhibiting the VEGF/VEGFR axis [81]. Since the RTK/VEGF receptor (VEGFR) axis induces the tumor angiogenesis in colorectal cancer, a study investigating the effects of EGCG on the activity of the VEGF/VEGFR axis and the expression of HIF-1α (both promote angiogenesis by elevating the VEGF levels) was undertaken for human colorectal cancer cells [82]. Within 3 h, the EGCG decreased the expression of HIF-1α protein, VEGF, HIF-1α, insulin-like growth factor (IGF)-1, IGF-2, epidermal growth factor (EGF) and heregulin mRNAs in SW837 colorectal cancer cells. These results express a constitutively activated VEGF/VEGFR axis, while a decrease was observed in the expression of VEGFR-2, p-VEGFR-2, p-IGF-1 receptor, p-ERK and p-Akt proteins within 6 h after EGCG treatment.

Experiments on concerning the uptake of EGCG solutions were indicative of their ability ot inhibit significantly the growth of SW837 xenografts in nude mice, a finding associated with the inhibition of VEGFR-2 expression and activation. The EGCG consumption was also found to inhibit the activation of ERK and Akt, both constituting the downstream signaling molecules of the VEGF/VEGFR axis and reducers of the VEGF-mRNA expression in xenografts [82]. These findings suggest that EGCG exerts growth inhibitory effects on colorectal cancer cells through the inhibition of VEGF/VEGFR axis activation that proceeds via the suppression of the HIF-1α and the expression of several major growth factors. Thus, EGCG might be useful in the chemoprevention and/or treatment of colorectal cancer.

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