Chapter 3

Phytocompounds Targeting Cancer Angiogenesis Using the Chorioallantoic Membrane Assay

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Additional information is available at the end of the chapter

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Abstract

Cancer is the second cause of mortality worldwide. Angiogenesis is an important process involved in the growth of primary tumors and metastasis. New approaches for controlling the cancer progression and invasiveness can be addressed by limiting the angiogenesis process. An increasingly large number of natural compounds are evaluated as angiogenesis inhibitors. The chorioallantoic membrane (CAM) assay represents an *in vivo* attractive experimental model for cancer and angiogenesis studies as prescreening to the murine models. Since the discovery of tumor angiogenesis, the CAM has been intensively used in cancer research. The advantages of this *in vivo* technique are in terms of low time-consuming, costs, and a lower number of sacrificed animals. Currently, a great number of natural compounds are being investigated for their effectiveness in controlling tumor angiogenesis. Potential reducing of angiogenesis has been investigated by our group for pentacyclic triterpenes, in various formulations, and differences in their mechanism were registered. This chapter aims to give an overview on a number of phytocompounds investigated using *in vitro*, murine models and the chorioallantoic membrane assay as well as to emphasize the use of CAM assay in the study of natural compounds with potential effects in malignancies.

Keywords: phytocompounds, tumor angiogenesis, chorioallantoic membrane assay

1. Introduction

Angiogenesis represents the process by which new vessels are formed from preexisting vessels [1] and has important implications associated with tumor growth and metastasis [2]. Studies

have shown that neovascularization is essential for tumor survival and growth, whereas in angiogenic absent conditions, tumor may display necrosis or even apoptosis [3, 4]. The angiogenic switch represents the process in which endothelial cells are led to a rapid growth state induced by stimuli secreted by the tumor microenvironment, comprising tumor and stromal cells, extracellular matrix components, immunologic cells, fibroblasts, adipocytes, muscle cells, and pericytes [5]. The switch may also involve downregulation of endogenous inhibitors of angiogenesis such as endostatin, angiostatin, or thrombospondin.

The undergoing of tumor angiogenesis represents a four-step process [6]: (i) tissue basement membrane injury; (ii) migration of endothelial cells, activated by angiogenic factors; (iii) endothelial cell proliferation and stabilization; (iv) continuous angiogenesis induced by angiogenic factors. Therefore, key elements in the angiogenesis process are the endogenous angiogenic factors. The most relevant angiogenic activators, signal mediators, and signaling effects are represented in **Figure 1**.

A class of proteins that is widely responsible for tumor angiogenesis is represented by growth factors, such as the vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet-derived endothelial growth factor (PDGF), tumor necrosis factor-α (TNF-α), epidermal growth factor (EGF), placental growth factor (PGF), transforming growth factor (TGF), granulocyte colony stimulating factor (GCSF), hepatocyte growth factor (HGF), angiostatin, and angiogenin [7]. However, VEGF is thought to be the main proangiogenic growth factor, because it induces all four phases of angiogenesis by augmenting vascular permeability, endothelial cell proliferation, endothelial cell migration, and capillary like tube formation [8]. Angiogenic cytokines or other growth factors such as VEGF are expressed under hypoxia conditions or by various oncogenes (e.g., mutant ras, erbB-2/HER2) [9].

As shown in **Figure 1**, after binding the tyrosine kinase specific domain of the receptors, multiple ways of signaling are possible for the angiogenic factors. Important molecular mechanisms involve activation of RAS/RAF1/kinase through the extracellular signal (ERK-1 și-2), inducing proliferation and differentiation; RAS/p38 mitogen-activated kinase (MAPK) and JUN/kinase 1-3 N-terminal, modulating inflammation, apoptosis, and differentiation; phosfatidyl-3-inositol kinase-1 (PI3K) and AKT dependent, regulating cell survival, mammalian receptor for rapamycin (mTOR), highly involved in proliferation and cell growth. Other inductor factors of the signaling pathways of angiogenesis are found in the cytoplasm (e.g., GAB1, SHC, SRC, PI3K, and phosfolipase γ C) [10].

VEGF and its receptors, the VEGFR family, remain intensively researched for targeting angiogenesis in different tumors. At the same time, other angiogenesis suppressing-related targets are being studied for the development of anticancer therapies for tumors resistant to anti-VEGF therapy. A number of therapeutic agents are currently in use for several malignancies: monoclonal antibodies against angiogenic growth factors (e.g., antibody against VEGF, Bevacizumab), inhibitors of angiogenic factors synthesis (e.g., mTOR inhibitor Rapamycin), and inhibitors of angiogenic factor receptors (tyrosine-kinase inhibitors, e.g., imatinib and sorafenib) [11]. Unfortunately, clinical response to the new molecular advances in cancer therapy by targeting angiogenesis is unsatisfactory. Resistance and low survival rates are signaled. New therapeutic approaches with minimal side effects are desired to act by targeting the multiple factors that are activated during tumor progression.

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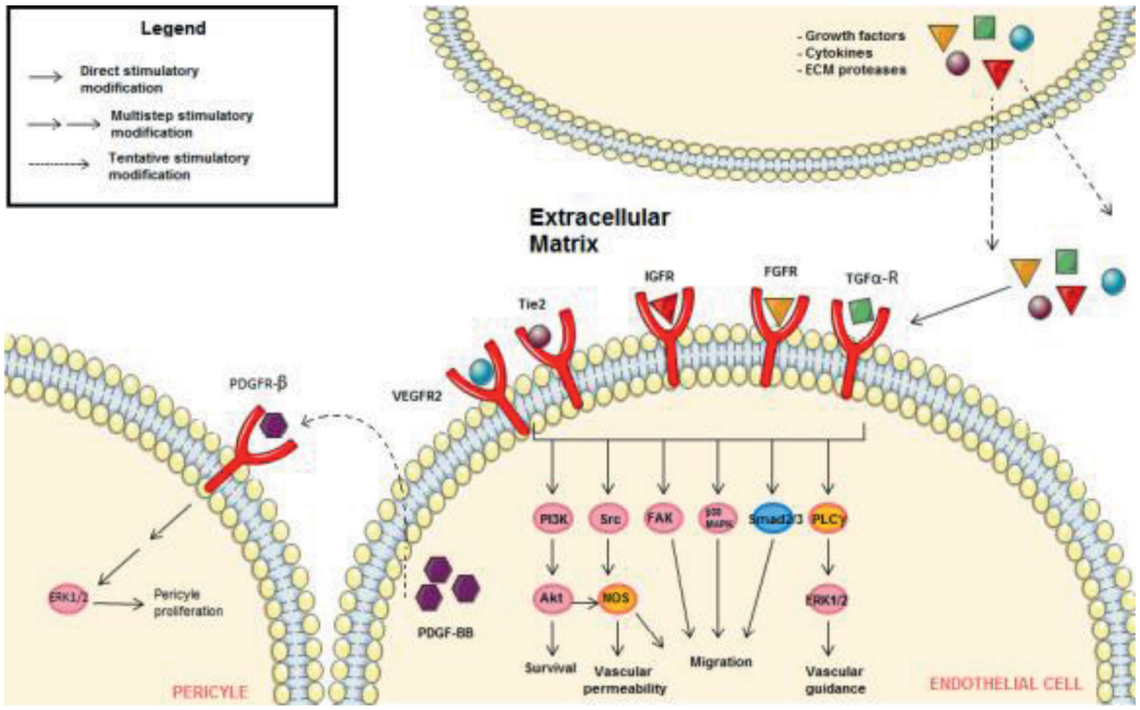

Figure 1. Angiogenic factors and signaling pathways involved in angiogenesis mediation. Abbreviations: Akt, RAC-alpha serine/threonine-protein kinase, ERK1/2, mitogen-activated protein kinase 1/2, FAK, focal adhesion kinase; FGFR, fibroblast growth factor receptor; IGFR, insulin growth factor receptor; MAPK, mitogen-activated protein kinases; NOS, nitric oxide synthase; p38, mitogen-activated protein kinase 11; PDGFR, olated-delivered endothelial growth factor receptor; PI3K, phosphatidylinositol 4,5-bisphosphate 3-kinase; PLCγ, phospholipase C gamma; Smad, Smad protein; Src, proto-oncogene tyrosine-protein kinase; TGFα-R, transforming growth factor α receptor; Tie, angiopoietin receptor; VEGFR, vascular endothelial growth factor receptor.

Based on the preventive effect that healthy diets have on the epidemiology of cancer, medicinal plants, spices, fruits, and vegetables represent an interesting source of phytochemicals. Natural compounds or even plant extracts are now considered important and accessible therapeutic or chemopreventive agents in cancer. In the search of the suitable phytocompounds to test for specific effects, virtual screening methods can be successfully applied in the selection of selective compounds for specific targets [12]. To avoid lack of selectivity, computational filtering schemes can be used [13]. Extensive studies demonstrate the high potential of plant-derived chemicals in controlling tumor angiogenesis with minimal secondary effects and drug resistance, by targeting multiple key pathways in a synergistic manner.

2. Experimental models for tumor angiogenesis: focus on the CAM assay

An important issue in angiogenesis studies is the appropriate choice of the assays. To evaluate the efficacy of potential phytocompounds and to identify potential targets within the angiogenic process, several methods both *in vitro* and *in vivo* can be applied. Each of them having one or more drawbacks, ideally more techniques are to be applied. *In vitro* techniques are used by co-culturing endothelial cell and other tumor microenvironment factors with tumor

cells in 2D or even 3D models which facilitate the identification of the involved molecular mechanisms. Despite the advances made in the direction of designing *in vitro* assays, the *in vivo* environment can be difficultly reproduced with such protocols [14]. To better assess the key aspects of tumor angiogenesis and therapeutic approaches, *in vivo* assays can be applied, such as the chick chorioallantoic membrane (CAM), the zebrafish, the sponge implantation, the corneal, or dorsal air sac and tumor angiogenesis models in rodents or rabbits [15]. Several drawbacks can still be cited, especially for the murine models, including high costs, complex technical and surgical abilities, and important quantities of test compounds.

2.1. Chorioallantoic membrane assay

The chorioallantoic membrane (CAM) assay represents an attractive *in vivo* experimental model for angiogenesis and cancer studies. The advantages of this *in vivo* technique in terms of costs, time, simplicity, reproducibility, and ease of the approval by the ethic committee make it a good prescreening assay to murine models in the research of biological systems and new therapeutic targets. Especially tumor angiogenesis and metastasis protocols benefit for a much shorter time for the tumor to grow and metastasize than the classical animal models.

The limitations of the model include a restricted number of reagents to work with due to low compatibility, nonspecific inflammatory reactions, keratinization of the membrane, and a vascular reaction that interferes with the visualization of vascular modifications. Technical skills may be significant to counteract these limitations [16, 17].

The chorioallantoic membrane is the vascularized respiratory extraembryonic tissue of avian species. First, this biologic system has been used for embryologic, immunological, and tumor grafting studies [18], and more recently, since the discovery of tumor angiogenesis [19], it is intensively applied in cancer research [20]. During the stages of embryo development, the immunologic, nervous, and nociceptive systems are not fully developed [21]. Several types of CAM assay protocols have been developed.

2.2. Uses in biological studies

The method can be applied for bioengineering development, morphology, biochemistry, transplant biology, cancer research, and drug development, but also in immunology, wound healing, tissue repair, or drug toxicity [22, 23]. The possibilities of imaging and evaluation have attracted many research studies. Nutritional therapeutics is an example of products approved by the U.S. Food and Drug Administration (FDA) that were preclinically evaluated in the CAM model [16].

Phytocompounds can be tested in order to evaluate their potential bioavailability, tolerability, and lack of irritation effects. For this purpose, the variations of the HET-CAM protocol can be applied, according the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommendations published in November 2006 in Appendix G of reference [24]. Our previous evaluations proved its applicability in testing different sets of compounds, i.e., surfactants and aflatoxins [25].

In the attempt of finding new means for cancer chemoprevention, the chorioallantoic membrane assay can be used to test natural compounds that could reduce or inhibit several pathways

involved in malignancies, especially pro-inflammatory cytokine activation and excessive angiogenesis. Tumor microenvironment, including inflammation and angiogenesis next to the development of new therapeutic targets for these pathological conditions, is intensively researched on murine models [26]. Previously, we have evaluated mast cell involvement in the angiogenesis process implementing a mastocytoma model on the CAM assay [27], which can be further developed for the evaluation of natural compounds on mast cells as key participants in the tumor microenvironment.

2.3. General *in ovo* method

Ex ovo or *in ovo* techniques are applicable. The *ex ovo* protocol involves the transfer of the egg content on day 3 of incubation into a Petri dish. It facilitates the visualization of the experiment, but the unnatural milieu of development of the embryo is detrimental to the survival rate of the specimens. Therefore, we prefer the *in ovo* protocol and is the type of method described here.

Fertilized eggs are horizontally incubated 7 days prior to use, at 37°C, in a controlled wet atmosphere. On the third day of incubation, in order to detach the chorioallantoic membrane, a volume of 2–3 ml of albumen is aspired through a perforation at the more pointed end of the eggs. The hole is resealed and returned to the incubator. The next day, a window is cut and resealed on the superior side of the shell. The eggs are returned to incubation until the day of the experiment [28]. Generally, 5–10 eggs are used for each test sample. Samples are applied inside a sterile plastic ring on the surface of the membrane. Samples are applied in triplicate. *In ovo* investigation by means of a stereomicroscope is performed throughout the experiment. Photographs are recorded for further analysis (**Figure 2**).

Starting with day 11 of incubation, samples can be considered active on excessive angiogenesis. The rapid growth of the vessels occurs during days 7–11; therefore, applying substances during this interval can be evaluated in terms of antiangiogenic effects. Morphometric evaluation of the angiogenic reaction can be conducted using a 0–5 arbitrary scale, the mean values expressing the vascular density around the site of application [20]. Finally, specimens are sacrificed and membranes are submitted to histological and immunohistological evaluation. On slides with immunohistochemical marked vessels, the mean microvascular density can be determined using the hotspot method, and counting the blood vessels, to calculate an antiangiogenic index, with the aid of the formula: $AAI = 1 - No_{BVtest}/No_{BVcontrol,}$ AAI = antiangiogenic index, BV = blood vessels [29].

2.4. Tumor angiogenesis model on CAM

Tumor cells are used on the CAM in order to obtain tumors, to study their microenvironment and the effects that phytochemicals might have. Tumor grafts can be used as well. Usually, cultured cancer cells are inoculated on the surface of the CAM, on day 10 of incubation, after being trypsinized and resuspended in culture medium to final concentrations in the range of 10^5–10^6 ml^{-1}. Cells can be applied directly on the CAM using a plastic ring for localizing the cells or using Matrigel impregnated with cells. Further, test compound solutions diluted with minimal DMSO (dimethyl sulfoxide) concentration in phosphate buffer can be applied on the

Figure 2. Chorioallantoic membrane assay—*in ovo* practical approach: incubation of the eggs (a–c); albumen removal, shell opening, and resealing (d–f); visualization of the CAM, sample application, and sample application inside a plastic ring (g–i) [30].

same spot as the cancer cell samples. *In ovo* stereomicroscopic follow-up is performed daily to register the changes in the vascular response around the tumor developing area that will be used for the morphometric analysis. On the final day of the experiment, after sacrificing the embryos, tumor masses are measured; the chorioallantoic membrane, the formed tumors, and some organs suspected to have metastasis are harvested and histologically processed.

In order to observe morphologic changes in the chorioallantoic membrane, hematoxylin eosin staining is analyzed. Different panels of immunohistochemical markers can be further applied: tumor cell markers and specific antibodies for different key proteins involved in the tumor microenvironment (e.g., endothelial cell marker-factor VIII, smooth muscle actin (SMA) marker, vascular endothelial growth factors, and its receptors, mast cells marker—Tryptase, the proliferation marker—Ki67). Results can reveal molecular modifications and serve to vascular density quantification.

Our experience is related to testing phytocompounds and plant extracts for the effect on angiogenesis. Using the angiogenesis method in the rapid stage of CAM development, we found that pentacyclic triterpenes, betulinic (BA) acid, and betulin (Bet) in various formulations with cyclodextrin and in nanoemulsion are potential antiangiogenic compounds, acting differently, both through direct and indirect mechanisms [31, 32]. Immunohistochemical staining for smooth muscle actin (SMA) on the specimens treated with betulin in nanoemulsion, next

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to blank and control samples, are shown in **Figure 3**. The low expression of the marker in the betulin-treated specimen indicates a minimal implication of pericytes in the angiogenesis process [32]. On the contrary, we found that betulinic acid determined rapid maturation of the vessels and high levels of SMA [31]. We also evaluated triterpenes and other types of natural compounds in melanoma models on CAM, which confirms the inhibitory effect on tumor angiogenesis (data not published).

Most studies that use the CAM assay are evaluated through stereomicroscopy that allows a series of quantitative measurements, and by histologic an immunohistological interpretation. Advances in the evaluation techniques include fluorescence microscopy, confocal microscopy, microCT scanning, and imaging, *in situ* hybridization (ISH), quantitative PCR (qPCR) determination of specific targets [16, 33].

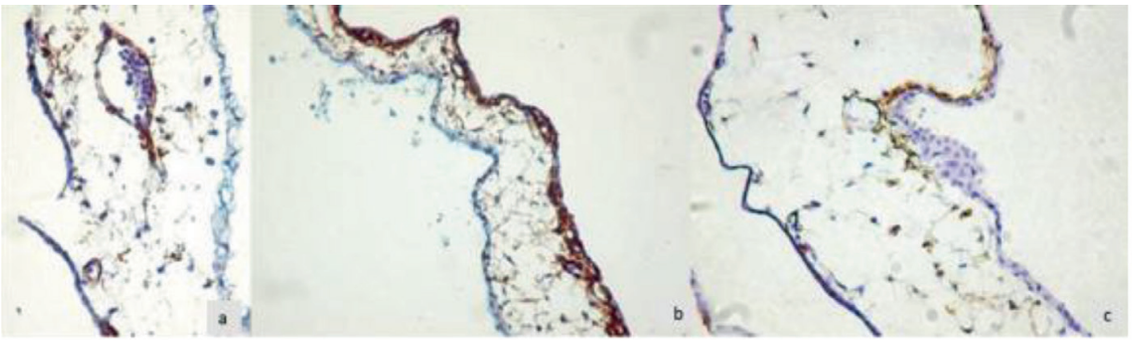
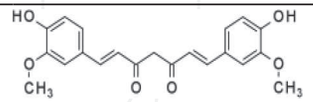
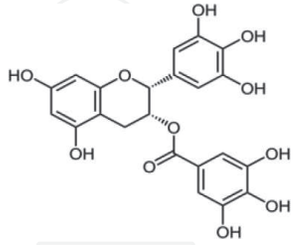
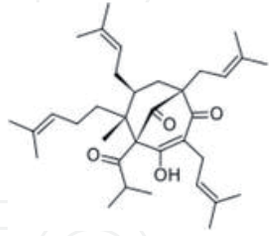
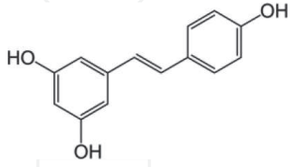
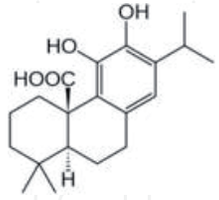
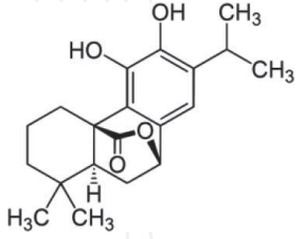
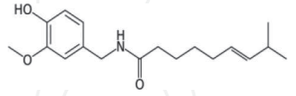
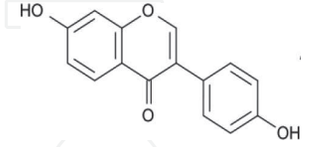
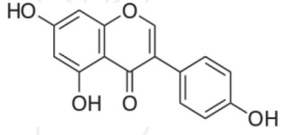


Figure 3. Light microscopic evaluation of CAM sections from ID 11 smooth muscle actin marker: (a) blank specimen, ×40, (b) control specimen treated with nanoemulsion, ×40, (c) specimen treated with betulin in nanoemulsion, ×40 [32].

3. Phytocompounds targeting cancer angiogenesis: *in vitro*, on the chorioallantoic membrane assay, in animal model

Chemicals derived from plant sources as well as various types of extracts have been already investigated for their effects on angiogenesis and on cancer. Currently, based on the failure of the approved therapeutics and also by crediting the traditional medicine philosophy that pathologies are imbalances that have to be rebalanced, the idea of multiple targeting through synergetic phytocompounds mixtures is gaining more attention. Extensive research is being dedicated to the understanding of their mechanism and their efficacy using *in vitro* and *in vivo* methods. The most in depth evidence comes from the results on cell cultures. *In vivo* methods also offer other accurate data on their effects. The chorioallantoic membrane assay is being used by more and more researchers for the evaluation of plant-derived chemicals or extracts. Correlations can be made using the results obtained for *in vitro*, animal and CAM assays, which will improve the knowledge and the future analysis to perform for the active compounds. We reviewed here some of the most investigated phytocompounds concerning the results obtained on all the three experimental models (**Table 1**).

Phytochemical class	Compound	Chemical structure	Plant source	*In vitro* effects	Effects on CAM	*In vivo* effects
Polyphenols	Curcumin		*Curcuma longa* L.	MiaPaCa-2; BxPC-3; Panc-1; MPanc-96 prostate cancer cell lines Reduced expression of NF-κB [34]	Angiogenesis inhibitor on small capillaries [35]	Athymic nude mice xenograft with prostate cancer cells Reduced expression of NFκB-p65, STAT3 and SRC; Reduced expression of ANXA2 and VEGFR2 [36]
	Epigallocatechin-gallate		*Camellia sinensis* L.	Hepatocellular carcinoma Inhibition of the VEGF-VEGFR axis [37]	Inhibition of fibroblast growth factor (FGF) and inhibition of mean branch formation and tumor weight of neuroblastoma-induced angiogenesis [38]	BGC-823 human gastric cancer xenograft mice model Reduction of VEGF [39]
Phloroglucinol derivative	Hyperforin		*Hypericum perforatum* L.	BAE—bovine aortic endothelial cell MDA-MB231 human breast cancer and NIH-3T3 mouse fibroblast cell Inhibition of capillary tube formation; Inhibition of urokinase and MMP2 [40]	Multiple target angiogenesis inhibitor [40]	Wistar rats inoculated with MT-450 at mammary tumor cells Suppression tumor-induced lymphangiogenesis [41]
Stilbene phytoalexin derivative	Resveratrol		*Vitis vinifera* L., *Polygonum cuspidatum* L.	YUZAZ6, M14, A375 melanoma cell lines Downregulation of VEGF and upregulation of TSP1 [42]	Significant reduction in angiogenesis in higher doses [43]	C57BL/6 Mice inoculated with Lewis lung carcinoma cells Inhibition of neovascularization [44]

Phytochemical class	Compound	Chemical structure	Plant source	*In vitro* effects	Effects on CAM	*In vivo* effects
Phenols	Carnosic acid		*Rosmarinus officinalis* L	HT-1080 Human fibrosarcoma cells, HL60 Human promyelocytic leukemia cells, HUVECs cells Inhibition of capillary tube formation; Decrease in the endothelial cells MMP-2 activity [45]	Antiangiogenic effect; emphasized activity for carnosic acid [45]	DMBA-induced hamster buccal Pouch carcinogenesis Suppressed expression of Cyclin D1 and NFκB; modulation of VEGF [46]
	Carnosol		*Rosmarinus officinalis* L	HT-1080 Human fibrosarcoma cells, HL60 Human promyelocytic leukemia cells, HUVECs cells capillary tube formation; Decrease in the endothelial cells MMP-2 activity [45]	Antiangiogenic effect; emphasized activity for carnosic acid [45]	n/a
	Capsaicin		*Capsicum* sp.	Hy-A549CoCl2-stimulated A549 lung cancer cells Inhibition of VEGF by downmodulation of HIF-1α; Increased p53 level [47]	Potent inhibitor of tumor-induced angiogenesis [48]	C57BL/6 mice Inhibition of VEGF and hemoglobin [48]
Isoflavones	Daidzein		*Trifolium pratense* L., *Glycine maxima* L.	LNCaP, PC-3, and DU-145 PCa cells - Down-regulation of ECGF1, FGF1, IGF1, FGFR3, IL-1β, IL-6, IL-8, PECAM1[49]	Antiangiogenic effect, anti-inflammatory effect with no membrane-irritating and toxic side effects[50]	n/a
	Genistein		*Trifolium pratense* L., *Glycine maxima* L.	BME cloned bovine microvascular endothelial cells Inhibition of bFGF [51]	Antiangiogenic effect, anti-inflammatory effect with no membrane-irritating and toxic side effects[50]	BALB/C nu/nu mice inoculated with Bel 7402 hepatocellular carcinoma Significant decrease of positive unit (PU) of the microvessels[52]

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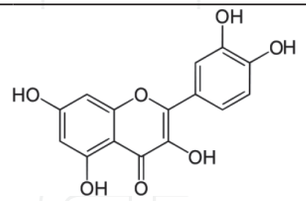
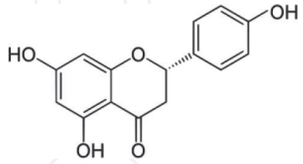
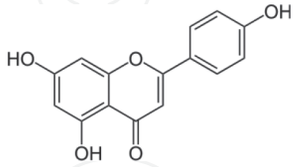
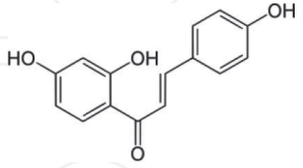
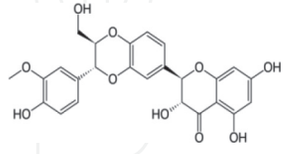
Phytochemical class	Compound	Chemical structure	Plant source	*In vitro* effects	Effects on CAM	*In vivo* effects
Flavonoids	Quercetin		*Camelia sinensis* L., *Angelica keiskei Momordica cochinchinensis*,	PC-3 prostate cells Inhibition of VEGF [53]	Potent angiogenesis inhibitor [53]	DMBA-induced experimental mammary carcinoma in rats Inhibition of H-ras protein; inhibition of VEGF and bFGF [54]
	Naringenin		*Citrus* sp.	Aspc-1 and panc-1 prostate cancer cells Inhibition of TGF-β1-induced migration; Decreased expression of MMP2 and MMP9 proteins [55]	Potent angiogenesis inhibitor [56]	n/a
	Apigenin		*Entada africana*, *Matricaria chamomilla* L	PC-3 and DU145 prostate cancer cells Inhibition of HIF-1α and VEGF LNCaP prostate cancer cells, HCT-8 colon cancer cells, and MCF-7breast cancer cells Inhibition of hypoxia-induced HIF-1α and VEGF[57]	Promising antiangiogenic effect [58]	BALB/cA-nu nude mice injected with PC-3 prostate cancer cells and OVCAR-3 ovarian cancer cells Inhibition of blood vessels formation; Inhibition of hemoglobin levels [57]
	Isoliquiritigenin		*Glycyrrhiza glabra* L	ACC-M, ACC-2 adenoid cystic carcinoma cells and EAhy926 endothelial hybridoma cell line Prevention of tube formation; Downregulation of VEGF [59]	Angiogenesis suppressor [60]	BALB/c nude mice injected with ACC-M cells Reduction in S6 phosphorylation; Decreased VEGF; Inhibition of the mTOR signaling pathway[59]
	Silibinin		*Silybum marianum* L	SW480, HT-29 and LoVo colorectal cancer cells Inhibition of NF-κB; Reduction of MMP9, COX-2 and VEGF[61]	dose-dependent suppressive on angiogenesis [62]	A/J mice with Urethane-induced lung tumors Inhibition of new microvessels formation; Decreased levels of IL-1α,-6, -9, -13, -16, IFN-γ and TNF-α[63]

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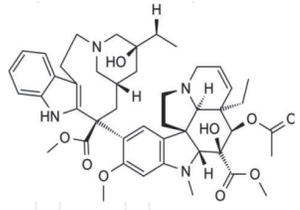
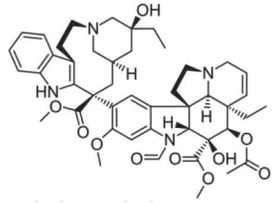
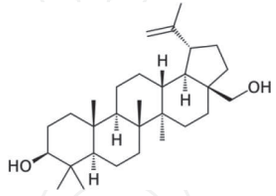
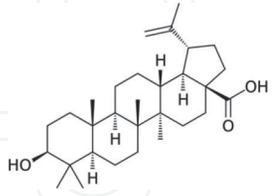
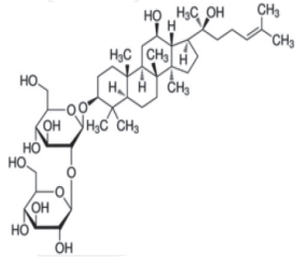
Phytochemical class	Compound	Chemical structure	Plant source	*In vitro* effects	Effects on CAM	*In vivo* effects
Alkaloids	Vinblastine		*Vinca* sp.	Human neuroblastoma cell lines Downmodulation of VEGF and VEGF-R2 [64]	Angiostatic activity [64]	Athymic (Nude-nu) mice injected with GI-LI-N cells Decrease of CD31-positive blood vessels; Downmodulation of VEGF and VEGF-R2[64]
	Vincristine		*Vinca* sp.	Glioblastoma cells—decreased expression of VEGF mRNA and the level of HIF-1α protein [65]	Antiangiogenic effects in neuroblastoma tumors in high doses [66]	Swiss nu/nu mice injected with Caki-l and Caki-2 renal carcinoma cells Inhibition of angiogenesis [67]
Pentacyclic triterpenes	Betulin		*Betula pendula, Prunus dulcis*	Apoptotic induction in MCF-7, A431 [68]	Strong direct antiangiogenic effects [32]	Balb/C mice DMBA/TPA skin carcinoma model Decreased expression of VEGF [32]
	Betulinic acid		*Betula pendula, Prunus dulcis*	SK-MEL2 melanoma and LNCaP prostate cancer cells - Decreased expression of Sp1, Sp3, Sp4, and VEGF [69]	Strong antiangiogenic effects [31]	Athymic nude mice with LNCaP cells as xenografts Tumor tissue less vascular; Decreased expression of Sp1, Sp3, Sp4, AR, and VEGF [69]
Tetracyclic Triterpenoid saponins	Ginsenoside Rg3		*Panax ginseng*	Eca-109—human esophageal carcinoma cell line and 786-0 renal cell carcinoma cell line Downregulation of VEGF expression via COX-2 pathway; Reduction of STAT3 phosphorylation; Decreased HIF-1α protein expression in Eca-109 cells[70]	Strong, multi-target inhibition of neovascularization, without affecting endothelial cell proliferation; lack of cytotoxicity [71]	C57BL/6 mice injected with LLC Lewis lung carcinoma cells Decreased tube formation of circulating progenitor cells; Suppression of VEGF dependent p38 and ERK signal pathways [72]

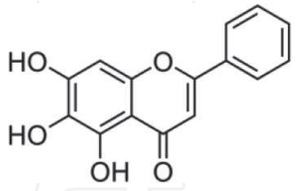
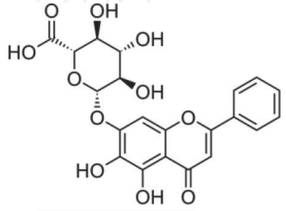
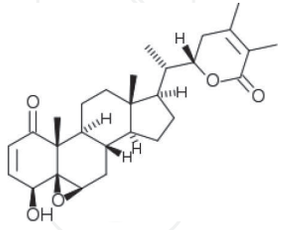
Phytochemical class	Compound	Chemical structure	Plant source	*In vitro* effects	Effects on CAM	*In vivo* effects
Flavones	Baicalein		*Scutellaria baicalensis* Georgi	H-460 cells assessed using BrdU assay Significant antiproliferative and pro apoptotic; inhibit bFGF-induced HUVEC tube formation in Matrigel stronger than baicalin [74, 75]	Dose-dependent antiangiogenic activity [74]	H-460 athymic nude mice, tumor growth and survival *low expression of* 12-LOX, *VEGF* and *FGFR-2 gene* [73]
	Baicalin		*Scutellaria baicalensis* Georgi	Growth and survival, MMP-2 expression, inhibit bFGF-induced HUVEC tube formation in Matrigel [74]; increases VEGF expression by activating the ERRα/PGC-1α pathway [75]	Dose-dependent antiangiogenic activity [74]	Inhibit growth of S180 solid tumor in mice [76]
Steroids	Withaferin A		*Withania somnifera* Dunal	Antiangiogenic activity in primary endothelial cells HUVEC [77]	Significant antiangiogenic activity [78]	Inhibits FGF-2 Induced angiogenesis in C57BL/6J mice [77]

Table 1. Common phytocompounds with *in vitro* and *in vivo* antiangiogenic activity.

4. Clinical trials correlation

Implementation of clinical trials is vital for the validation and future use of the active phytocompounds as additional therapies to the oncologic protocols or as chemopreventive strategies. These types of experiments are difficult to implement and therefore not many trials are finalized for the evaluation of antiangiogenic effect in cancer. Two of the above-listed phytochemicals (**Table 1**) benefit from large investigations among which some are clinical trials, but the modulation of the angiogenic process does not appear as a distinct evaluation, cancer effects being the first ones to be described.

Most of the controlled clinical trials of curcumin supplementation in cancer patients aimed to determine its feasibility, tolerability, safety, and to provide early evidence of efficacy [79]. For patients with advanced colorectal cancer, oral doses up to 3.6 g/day for 4 months were well tolerated, although the systemic bioavailability of oral curcumin was low [80]. For this dose, trace levels of curcumin metabolites were measured in liver tissue, but curcumin itself was not detected [81]. These findings suggested that oral curcumin is effective as a therapeutic agent in cancers of the gastrointestinal tract. Other trials found that combining curcumin with anticancer drugs like gemcitabine in pancreatic cancer [82], docetaxel in breast cancer [83], and imatinib in chronic myeloid leukemia may confer additional benefits to conventional drugs against different types of cancer.

Green tea made from *Camellia sinensis* L. leaves, originated in China, is one of the most extensively consumed beverages and achieved significant attention due to health benefits against cancer. Representative compounds are polyphenols and catechins with therapeutic potential against cancer [84]. Recent clinical trials proved that green tea extract and epigallocatechin gallate (EGCG) can be active in several forms of cancer. There is an increasing trend to employ green tea extract and EGCG as conservative management for patients diagnosed with less advanced prostate cancer. Combinations of chemopreventive agents should be carefully investigated because mechanisms of action may be additive or synergistic [85]. Several clinical examinations reported different molecular mechanisms regarding green tea beneficial effects against oral cancer chemoprevention [86–88]. Lung cancer induction may also be inhibited by tea polyphenols. Some studies suggest that individuals who never drank green tea have an elevated lung cancer risk compared to those who drank green tea at least one cup per day, and the effect is more pronounced in smokers [88]. Hepatocellular carcinoma (HCC) usually develops in a cirrhotic liver due to hepatitis virus infection. Green tea catechins (GTCs) may possess potent anticancer and chemopreventive properties for a number of different malignancies, including liver cancer. Antioxidant and anti-inflammatory activities are key mechanisms through which GTCs prevent the development of neoplasms, and they also exert cancer chemopreventive effects by modulating several signaling transduction and metabolic pathways where angiogenesis is exacerbated. Several interventional trials in humans have shown that GTCs may ameliorate metabolic abnormalities and prevent the development of precancerous lesions [89].

5. Conclusion

Currently, a great number of natural compounds are being investigated for their potential effectiveness in controlling tumor angiogenesis and therefore the reduction of tumor growth and metastasis. Observing the high number of molecular pathways that are deregulated in tumor angiogenesis and that many phytocompounds are active on several key factors, it is recommendable that more *in vivo* studies should investigate mixture of compounds for broader targeting, having eventually lower secondary effects and resistance. The optimal experimental technique is an important factor in order to get a useful output. More types of assays are always a good choice, including *in vivo* assays. The chorioallantoic membrane protocol is a good candidate for one type of "golden standardized method" in tumor angiogenesis, being a versatile, rapid, easy, and cheap method to apply in the research of phytocompounds. A great number of plant-derived chemicals, alone or in combination, are studied using this method, but standardization, next to applying new analysis techniques will outcome useful data that will be easier translated to clinical trials.

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