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Combination of Epigallocatechin-3-gallate and Silibinin: A Novel Approach for Targeting Both Tumor and Endothelial Cells

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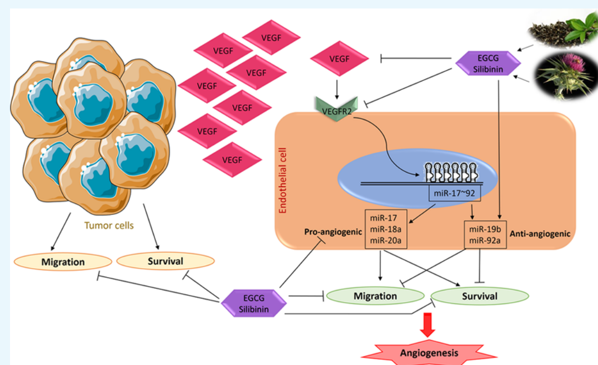
Supporting Information

ABSTRACT: Despite promising benefits, anti-angiogenic strategies have revealed several drawbacks, which necessitate development of novel approaches in cancer therapy strategies including non-small-cell lung cancer, as one of the leading causes of cancer death, all over the world. Combination of flavonoids could be a safe and effective option to synergize their impact on mechanisms controlling tumor angiogenesis. In this study, we have investigated the plausible synergism of epigallocatechin-3-gallate (EGCG) and silibinin on endothelial cells, for the first time. Cell viability and migration were evaluated by survival and wound healing assays, respectively. Then, we assessed the expression of *VEGF*, *VEGFR2*, and miR-17−92 cluster using real-time polymerase chain reaction in endothelial−tumor cell and endothelial−fibroblast coculture models. EGCG ± silibinin suppressed endothelial and lung tumor cell migration in lower than 50% toxic doses. *VEGF*, *VEGFR2*, and pro-angiogenic members of the miR-17−92 cluster were downregulated upon treatments. Specifically, the combination treatment upregulated an anti-angiogenic member of the cluster, miR-19b. Our data provides evidence to utilize the EGCG and silibinin combination as a novel approach to target tumor angiogenesis in the future.



INTRODUCTION

Since its first suggestion in 1971, anti-angiogenic therapy of cancer has been known as an essential approach in treating many types of the disease.[1] In non-small-cell lung cancer (NSCLC), like other solid tumors, angiogenesis is accompanied with highly invasive and metastatic properties of disease. Treating approaches including anti-angiogenic agents are among the promising therapies in clinical trials. However, there is a critical need for more studies in this area to overcome observed toxicities and drawbacks.[2] Angiogenesis, the process of creating new blood vessels from preexisting ones, is a key physiological and developmental event to maintain homeostasis. Disrupted regulation of angiogenesis is directly linked to different pathologies and life-threatening diseases, particularly cancer.[3] Regardless of the angiogenesis-inducing source, endothelial cell functions such as survival and migration are crucial in angiogenesis. Endothelial cell survival and migration directly depend on vascular endothelial growth factor (VEGF)-mediated pathways, the key target in most available anti-angiogenic therapeutic options.[4] On targeting this growth factor, its receptors or downstream mediators, by current approaches, not only inhibit tumor angiogenesis but also cause a systemic endothelial cell dysfunction and subsequent toxicity and cardiovascular diseases.[5] In addition, resistance to VEGF-targeted therapies has been reported in clinical settings.[6] Therefore, development of novel and safe strategies is an unmet health priority to minimize the side effects of anti-angiogenic therapeutics.

Tumor microenvironment is a mixture of different factors, secreted from cancer and stromal cells in response to detrimentally imposed conditions.[7] The angiogenic response is a consequence of the interplay among several angiogenesis inducers and inhibitors, secreted by the cells in a tumor microenvironment. However, targeting a single molecular pathway in a specific cell type would not suffice the treatment goals.[4b,8] Thus, finding new treatment options targeting multiple target(s) that effectively inhibit the pathological angiogenesis with minimal disruption of physiological angiogenesis is a crucial need.[7b,9]

MicroRNA (miRNA)s are a class small non-coding RNAs that negatively regulate their downstream target genes either by degrading the mRNA or inhibiting its translation to the protein.[10] MiRNAs play diverse roles in endothelial cell integrity and functions. Importantly, they control angiogenesis by regulation of target genes involved in endothelial cell migration and survival.[11] Several lines of evidence indicate contribution of the highly expressed polycistronic miR-17−92 cluster to angiogenic properties and tumor development.[12]

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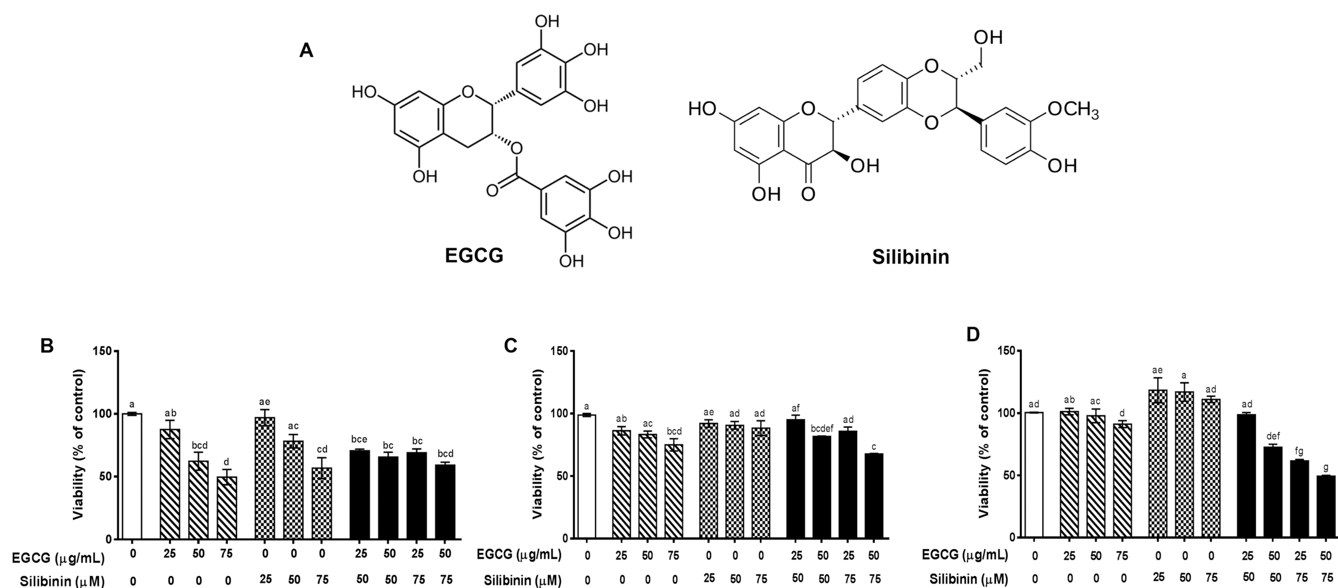

Figure 1. Structural properties and the effect of epigallocatechin-gallate (EGCG, $C_{22}H_{18}O_{11}$) and silibinin ($C_{25}H_{22}O_{10}$) on cell viability. (A) Structural properties of EGCG and silibinin. Relative cell viability of the human umbilical vein endothelial cell (HUVEC) (B), A549 (C), and human dermal fibroblasts (HDFs) (D) was evaluated in response to EGCG (25, 50, and 75 μgr/mL), silibinin (25, 50, and 75 μM), and combinations (25 μg/mL EGCG + 50 μM silibinin, 50 μg/mL EGCG + 50 μM silibinin, 25 μg/mL EGCG + 75 μM silibinin, 50 μg/mL EGCG + 75 μM silibinin) compared with control, in 24 h. Values represent mean ± standard error of the mean (SEM) of at least three replicates. (Dissimilar letters indicate significant difference with max $P < 0.05$.)

Furthermore, it has been shown that the miR-17−92 cluster regulates the endothelial cell function and angiogenic switch upon VEGF induction.[13] This suggests the miR-17−92 cluster to be a potential target in treating malignancies through affecting both tumor and endothelial cells.

A compelling body of evidence support the notion that flavonoids could be considered as promising treatment options to combat cancer. Particularly, in the context of angiogenesis, pleiotropic activity of these phytochemicals via targeting multiple molecular pathways in either tumor or endothelial cells interferes with tumor development and angiogenesis. Combinatorial application of different flavonoids is plausible to strengthen their anti-angiogenic capacity.[14] Epigallocatechin-3-gallate (EGCG)[15] and silibinin[16] are the major flavonoid-type active constituents of two of the most consumed plant products, green tea and milk thistle (*Silybum marianum*), modulating cell proliferation and apoptosis induction, the anti-angiogenic and anticancer effects of which have been reported in a variety of tumors (Figure 1A).[14,17]

In this study, we have investigated the impact of combining these flavonoids on cell viability and migration of human umbilical vein endothelial cell (HUVEC) and A549, an epithelial carcinoma cell line, which is a common model for non-small-cell lung cancer (NSCLC) studies. Moreover, we have evaluated the differential effect of single versus combined treatments of EGCG and silibinin on gene expression changes of *VEGF* and *VEGFR*2 and their downstream miR-17−92 cluster to find out the potential mechanism underlying the pleiotropic activity of these secondary metabolites. We have focused on the potential additive anti-angiogenic effect of EGCG and silibinin considering the ease of access and extensive consumption worldwide.[18] More importantly, EGCG[15b] and silibinin[19] have been previously reported to play anti-angiogenic roles individually. However, the synergistic effect of EGCG and silibinin co-treatment on endothelial cell mechanisms underlying angiogenesis has remained to be addressed.

RESULTS AND DISCUSSION

EGCG and Silibinin Treatment Regulates Viability in Endothelial and Lung Tumor Cells and Fibroblasts. Previous studies have reported the inhibitory effect of EGCG and silibinin on cell viability of multiple cells.[16b,20] However, the potential synergistic effect of EGCG and silibinin treatment on cell viability has remained to be addressed. Our findings indicate that EGCG (25−75 μg/mL) is able to significantly inhibit the viability of HUVEC in 24 h not exceeding 50% of the control group; >60% viability was observed in response to 50 μg/mL (Figure 1B). These results are consistent with previous studies indicating a dose-dependent decrease in cell viability in response to EGCG. In the context of EGCG, regulation of Wnt and Id signaling pathways has been suggested as the anti-proliferative mechanism of action in HUVEC.[20b] Wnt signaling is involved in angiogenesis through regulation of cell proliferation, survival, migration, differentiation, and apoptosis.[21] Regulation of key angiogenesis genes such as *VEGF* has been shown as a target of Wnt signaling,[22] which could be considered in our future investigations. Other studies have reported no significant change in HUVEC viability 24 h after treatment with EGCG (50 μM, ∼23 μg/mL), which are analogous to our data at 25 μg/mL.[23]

We observed a decreasing but not significant trend in cell viability of HUVEC in response to silibinin treatments (25−75 μM). As previously shown, this reduction could be relevant to a pleiotropic activity of silibinin on endothelial cells. Increase in Cip1/p21, Kip1/p27, and p53 and subsequent cell cycle arrest and apoptosis induction through upregulating BAX and downregulating Mcl1, on one hand, and suppressing Akt and necrosis factor-κB (NF-κB) signaling, on the other hand, are the plausible pathways that are implicated in silibinin effect on

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endothelial cells.[16a] The converging result of P53 induction[24] and reduction of Akt[25] and NF-κB[26] is downregulation of VEGF, which can be proposed as the downstream mechanism of silibinin action on endothelial cells. Vakili Zahir et al. have reported a higher tolerance of HUVEC to silibinin treatment compared with the HepG2 (human hepatocellular liver carcinoma) cell line, though treatment with a high level of silibinin leads to a necrotic cell death in HUVEC.[27] This indicates that different tumor cell lines, liver versus lung, may differently respond to silibinin.

Interestingly, our results revealed that the combination of EGCG and silibinin at the same concentrations led to no significant reduction of cell viability of HUVEC in comparison with single treatments at equal time point (Figure 1B), and cell viability of HUVEC following the EGCG (50 μg/mL) and silibinin (50 μM) combination treatment was nearby 70%. The importance of this finding is that co-treatment of these two flavonoids enhanced cytotoxicity in lung tumor cells compared with single treatments (Figure 1C).

As shown in Figure 1C, viability of the malignant lung tumor cell line, A549, was not significantly influenced upon 24 h treatment with EGCG (25 and 50 μg/mL) or silibinin (25, 50, and 75 μM). In contrast, the combination of EGCG (50 μg/mL) and silibinin (50 and 75 μM) significantly reduced A549 cell viability, not exceeding 60% of the control group.

A growing number of studies have shown the apoptosis induction and inhibitory activities of EGCG on the growth and development of cancer cells including head and neck,[28] breast,[29] colorectal,[30] prostate,[31] hepatocellular carcinoma,[32] Kaposi's sarcoma,[33] and lung cancer cells.[20a] Importantly, it has been shown that A549 cells are extremely resistant to EGCG treatment in vitro.[34] However, high concentrations of EGCG are capable of inducing apoptosis in these cells.[35] Similarly, there are plenty of studies indicating the suppressive effect of silibinin on hepatocellular carcinoma,[36] prostate,[37] breast,[38] neuroblastoma,[39] colorectal,[40] and lung[16b] cancer cells. Silibinin treatment interferes with cell growth prominently through G1 arrest[16b] and apoptotic induction[41] in NSCLC. Our results showed no significant change in cell viability of A549, as a NSCLC model, upon EGCG or silibinin single treatment at lower doses, which was reverted by increasing the concentration (Figure S1). The significant increase in toxicity against A549 cells through co-treatment with both components is suggestive of a direct tumor-killing activity, whose precise underlying mechanism needs to be unfolded.

In parallel, we have evaluated the fibroblast response to relevant concentrations of EGCG or silibinin. Proliferation of fibroblasts is important in wound healing as an example of physiological angiogenesis. They contribute to new vessel formation and integrity by secreting extracellular matrix components.[42] We showed that 24 h treatment with EGCG (25 and 50 μg/mL) or silibinin (25, 50, and 75 μM) did not significantly affect the viability of normal fibroblast in comparison with the control group (Figure 1D). However, treatment with mixed concentrations of EGCG (50 μg/mL) and silibinin (50 and 75 μM) revealed a significant decrease in cell viability not exceeding 50%; HDFs revealed higher than 70% viability following treatment with EGCG (50 μg/mL) and silibinin (50 μM). Our results suggest that normal fibroblasts are resistant to EGCG or silibinin; however, combination treatment moderates their viability not more than 50% of untreated cells. It is consistent with the previously reported noncytotoxic effect of EGCG or silibinin on normal fibroblasts.[43]

As determined by our half-maximal inhibitory concentration (IC_{50}) measurements, HUVEC was the most sensitive cell to EGCG or silibinin treatment (Table 1). In fact, IC_{50} values of both components were significantly lower in HUVEC than those in A549 and normal fibroblasts in 24 h. Collectively, EGCG and silibinin induced a dose-dependent decrease in cell viability, which was further confirmed at higher concentrations (Figure S1, Supporting Information).

Table 1. IC_{50} Values of EGCG or Silibinin in HUVEC, A549, and HDF Cells

	HUVEC	A549	HDF
EGCG (μg/mL)	68.07 ± 1.87	444.4 ± 1.71	3190 ± 2.50
silibinin (μM)	91.22 ± 1.64	381.8 ± 1.96	260.3 ± 1.98

Endothelial and Lung Tumor Cell Migration Is Markedly Reduced upon EGCG and Silibinin Treatment. Cell migration is a key process not only in angiogenesis but also in tumor metastasis. It is a process by which endothelial cells undergo massive angiogenesis under not only physiological but also pathological conditions, e.g., tumor growth.[44] We conducted the wound healing assay to investigate the potential inhibitory effect of the EGCG and silibinin combination on endothelial and tumor cell migration in concentrations at which beyond 50% cell viability was observed because high toxicity would contradict with the foundation of cell migration estimation. Our results approved the previous reports on the inhibitory effect of EGCG[23a] or silibinin[16a] on HUVEC migration. Enumerating migrated cells to the wound area using imageJ revealed that migration of HUVEC is significantly suppressed in response to noncytotoxic doses of EGCG or silibinin in a dose-dependent manner. Importantly, we found that the antimigratory effect of EGCG or silibinin significantly elevated upon combination treatment of these flavonoids in HUVEC, demonstrating a remarkable synergistic effect of EGCG and silibinin on HUVEC migration (Figure 2).

Wang et al. have shown that the EGCG-induced antimigratory effect on HUVEC is mediated by suppression of tumor necrosis factor (TNF)-NF-κB axis.[23b] A downstream mechanism of suppressing NF-κB in cell migration is reduction in the *VEGF* expression as a regulatory target for EGCG and silibinin treatment in our study.

Migration is a critical step in cancer cell invasion and metastasis.[45] In the context of lung tumor cells, EGCG[46] or silibinin[47] is capable of inhibiting cell migration. Similar to HUVEC, treatment with EGCG or silibinin alone inhibited migration of A549 tumor cells compared to the control untreated group. As a novel finding, we report for the first time that the combination of EGCG and silibinin is more potent to attenuate migration of A549 cells, as a typical NSCLC model, compared to either EGCG or silibinin alone. We observed that the combination of EGCG (25 and 50 μg/mL) and silibinin (50 and 75 μM) significantly declined migration of A549 tumor cells compared with the treatment with corresponding concentrations of each flavonoid (Figure 3). It should be noted that co-treatment with EGCG (50 μg/mL) and silibinin (50 μM) led to the highest inhibitory effect on A549 cell migration compared to that of other concentrations examined. Therefore,

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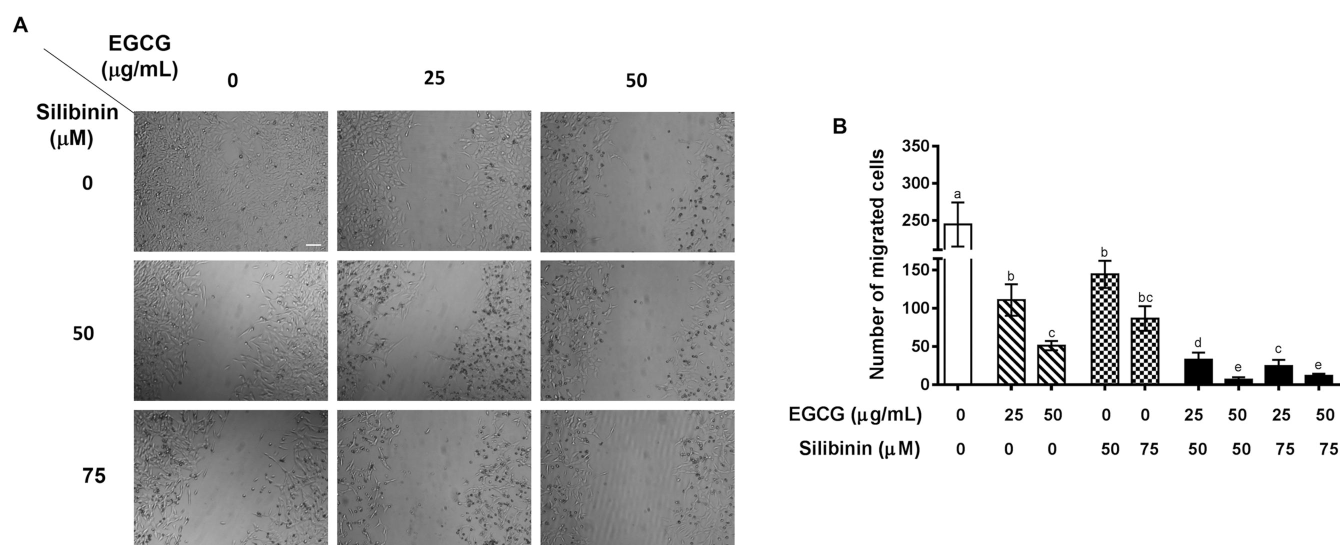

Figure 2. EGCG and silibinin inhibit HUVEC migration. (A) Cell migration effects of EGCG (25 and 50 μg/mL), silibinin (50 and 75 μM), and their combination (25 μg/mL EGCG + 50 μM silibinin, 50 μg/mL EGCG + 50 μM silibinin, 25 μg/mL EGCG + 75 μM silibinin, 50 μg/mL EGCG + 75 μM silibinin) in 24 h on HUVEC cells. (B) Representative indication of cell migration response of HUVEC to EGCG, silibinin, and their combination. Number of cells within four randomly chosen wound regions were measured using ImageJ and were normalized to the control group (scale bar: 100 μm). Dissimilar Letters indicate significant difference, with max $P < 0.05$, using statistical analysis by one-way analysis of variance (ANOVA), and values represent mean ± SEM.

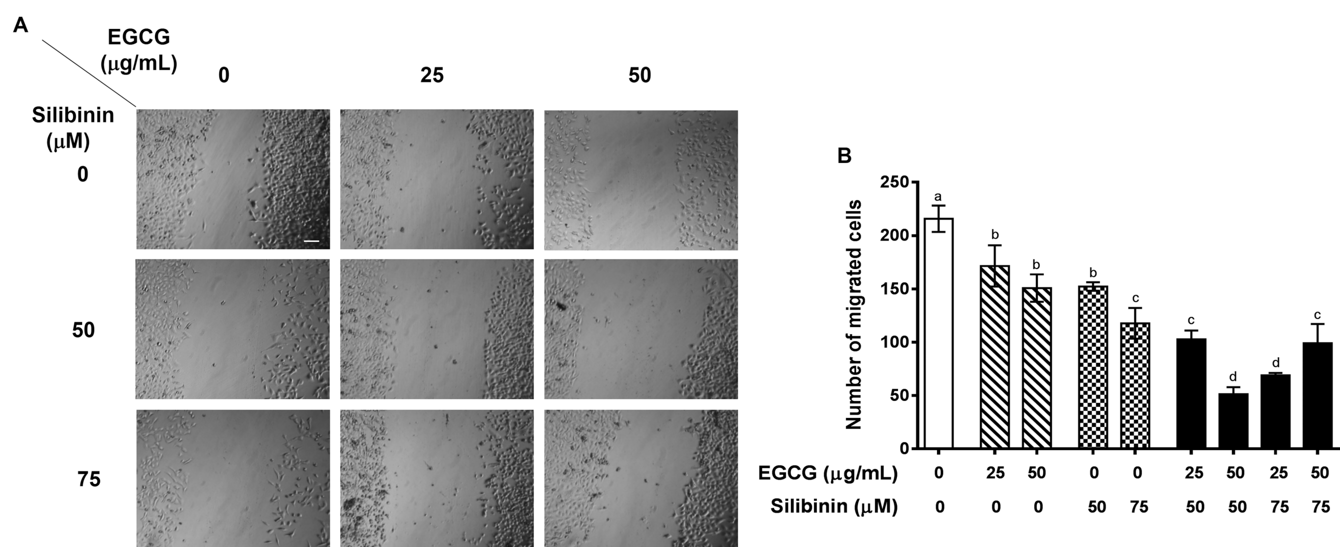

Figure 3. EGCG and silibinin inhibit A549 cell migration. (A) Cell migration effects of silibinin (25, 50, and 75 mM), EGCG (25 and 50 mg/mL), and their combination (25 μg/mL EGCG + 50 μM silibinin, 50 μg/mL EGCG + 50 μM silibinin, 25 μg/mL EGCG + 75 μM silibinin, 50 μg/mL EGCG + 75 μM silibinin) in 24 h on HUVEC cells. (B) Representative indication of cell migration response of A549 to EGCG, silibinin, and their combination. The number of cells within four randomly chosen wound regions were measured using ImageJ and were normalized to the control (scale bar: 100 μm). Dissimilar Letters indicate significant difference, with max $P < 0.05$, using statistical analysis by one-way ANOVA, and values represent mean ± SEM.

these doses were utilized in our mechanistic gene expression studies.

Altogether, our wound healing data suggest that the combinatorial treatment of EGCG and silibinin exerts a synergistic effect on inhibition of migration both in tumor and endothelial cells. While 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) results indicated cell viability around 70% in HUVEC and higher than 80% in A549, following EGCG (50 μg/mL) and silibinin (50 μM) combination administration, the antimigratory capacity of equal treatment was greater than 80% of the control group in both cell lines.

Inhibitory Effect of EGCG and Silibinin Treatment on Endothelial Cell Migration and Viability Is Mediated via Downregulation of VEGF−VEGFR2 Axis. Next, we aimed to determine the mechanisms underlying regulation of cell viability and migration by EGCG and silibinin. It has been previously shown that the VEGF pathway is the key component of angiogenesis in various contexts.[3] VEGF is the master mediator in both physiological and pathological angiogenesis.[48] VEGF−VEGFR2 signaling critically regulates endothelial cell survival, proliferation, migration, and tube formation.[49] The VEGF pathway has emerged as a specific target to minimize elevated angiogenesis in various types of

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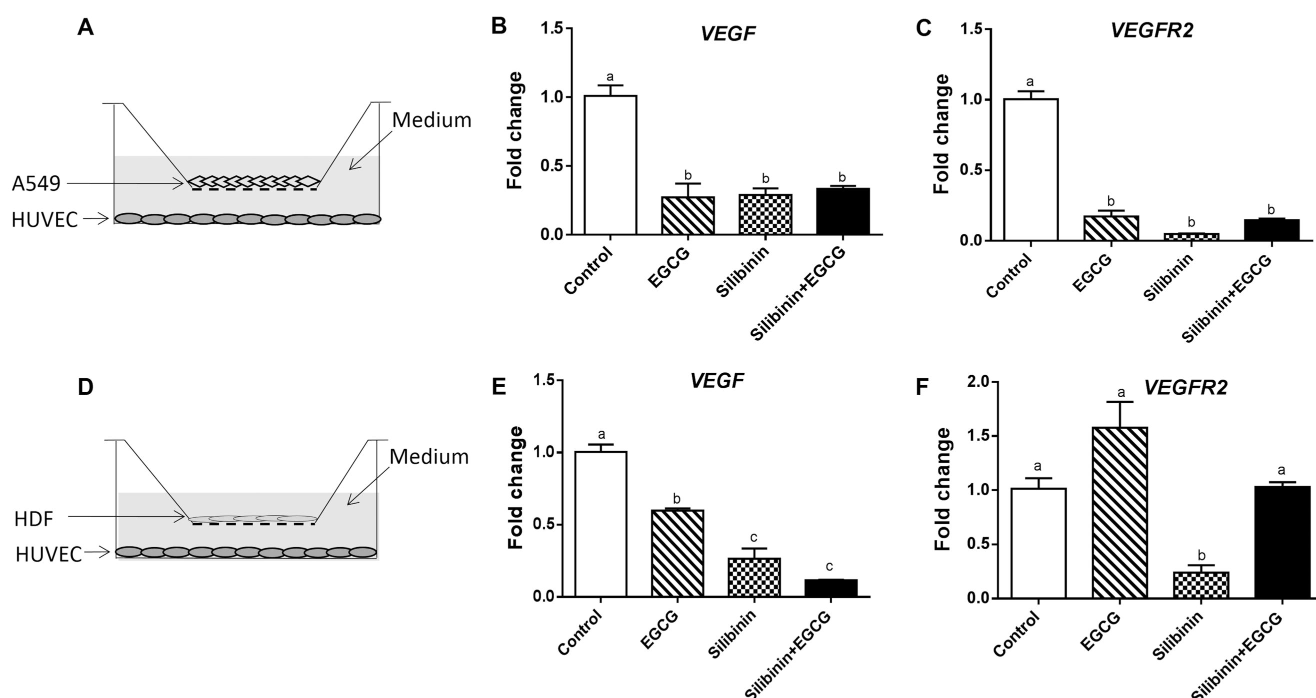

Figure 4. Gene expression changes of *VEGF* and *VEGFR2* in HUVEC cells cocultured with A549 or HDF. Representative of HUVEC cocultured differentially with A549 (A) or HDF (D). RNA extraction was performed from HUVEC after 24 h of treatment with EGCG (50 μg/mL), silibinin (50 μM), or the combination (50 μg/mL EGCG + 50 μM silibinin). *VEGF* (B) or *VEGFR2* (C) expression in HUVEC cocultured with A549. *VEGF* (E) or *VEGFR2* (F) expression in HUVEC cocultured with HDF. Gene expression changes were normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Values represent mean ± SEM of at least two replicates. (Dissimilar letters indicate significant difference with max $P < 0.05$.)

cancer.[50] It has been reported that green tea extract is capable of diminishing the expression of *VEGF* and its receptors *VEGFR1* and *VEGFR2*.[51] Deep et al. have shown the downregulation of *VEGF* and *VEGFR2* in HUVEC following treatment with milk thistle-derived flavonolignans including silybin A, silybin B, isosilybin A, and isosilybin B.[20c] To our knowledge, there is no study on the specific effect of EGCG or silibinin on the VEGF axis in endothelial cells, so far. In this study, we have investigated the impact of EGCG, silibinin, and their combination on the expression of *VEGF* and *VEGFR2* in HUVEC. The novelty of our study is evaluation of gene expression changes provoked by EGCG and silibinin in HUVEC in the presence of A549 tumor cells as robust inducers of angiogenesis. The angiogenic function of endothelial cells is influenced by tumor cells or fibroblasts. Therefore, we examined whether the effect of EGCG and silibinin on endothelial cells is mediated by altering the function of these cells or not. First, HUVECs were cocultured with A549 tumor cells and then treated with EGCG, silibinin, or their combination for 24 h (Figure 4A). Gene expression analysis in isolated HUVEC demonstrated that EGCG, silibinin, and their combination dramatically downregulated *VEGF* (Figure 4B) and *VEGFR2* (Figure 4C) as master mediators of angiogenesis. We further showed that *VEGF* expression is significantly lowered in HUVEC cocultured with primary human fibroblast obtained from healthy individuals, in response to EGCG, silibinin, or their combination (Figure 4D,E). EGCG treatment was not able to significantly reduce *VEGFR2* expression in HUVEC upon coculture with normal fibroblast. In contrary, silibinin reduced *VEGFR2* expression, which was reversed after co-treatment with EGCG (Figure 4E).

These data are suggestive of an EGCG ± silibinin-induced *VEGF*−*VEGFR2* signaling in endothelial cells cocultured with tumor cells. However, the nonsignificant effect of the EGCG and silibinin combination on endothelial cells cocultured with normal fibroblasts indicates a selective beneficial impact of this treatment under healthy conditions, which should be mechanistically unfolded in the future.

Expression of miR-17−92 Cluster Is Tightly Regulated by EGCG and Silibinin Treatment. To decipher the consequence of diminished expression of *VEGF*, we evaluated endothelial changes of miR-17−92 expression in the presence of tumor cells. It has been reported that VEGF induces the expression of the polycistronic miR-17−92 cluster, which is critically involved in endothelial cell function, angiogenesis, and tumor metastasis via regulation of VEGF−VEGFR2 expression.[13] The polycystronic miR-17−92 cluster encodes six miRNAs, namely, miR-17, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92a, which are crucially implicated in tumor angiogenesis. Upregulation of the miR-17−92 cluster leads to increased angiogenic and invasive properties of the tumor cells.[12] MiR-17−92 is highly expressed in endothelial cells, and it has been revealed that each member acts differentially on angiogenesis.[52] For instance, miR-18a and miR-20a[53] promote angiogenesis in contrary to miR-19b[54] and miR-92a[55] that induce anti-angiogenic features. To our knowledge, the regulatory role of EGCG and silibinin in regulation of miR-17−92 expression has not been investigated before. Thus, we performed quantitative real-time polymerase chain reaction (qRT-PCR) to evaluate gene expression changes of the miR-17−92 cluster in HUVEC following treatment with EGCG, silibinin, or their combination cocultured with A549 cells or normal fibroblast.

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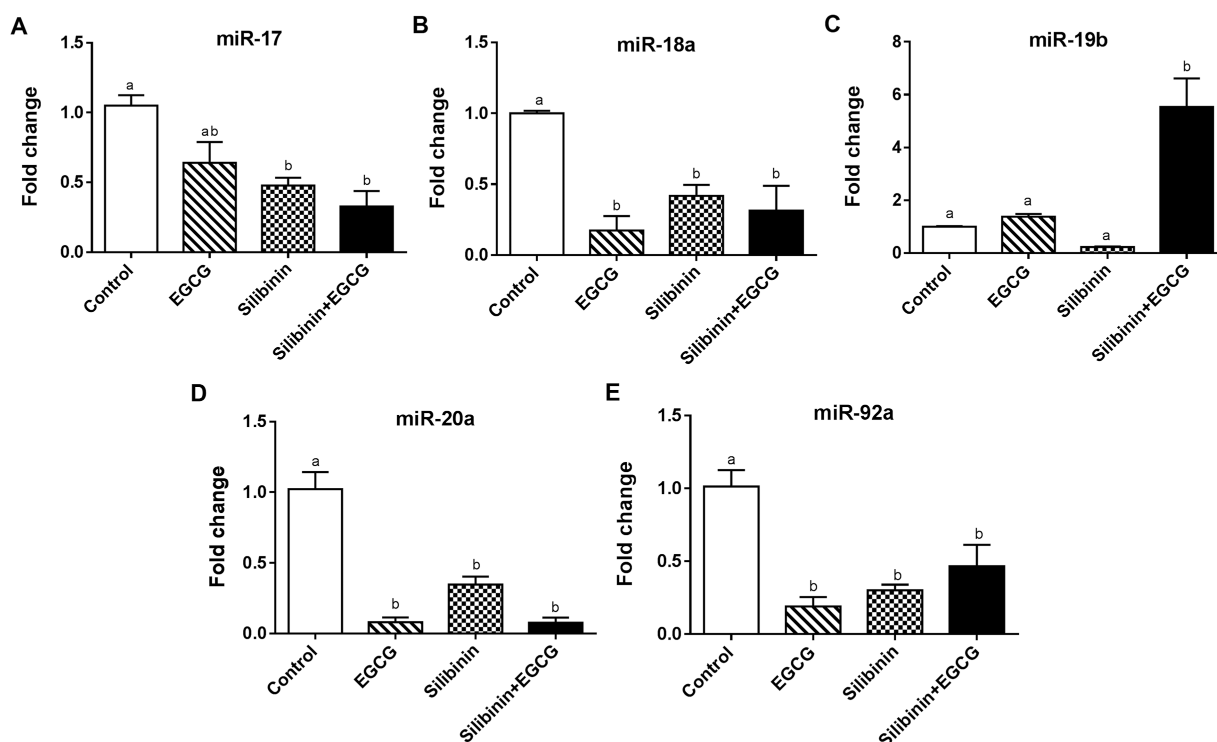

Figure 5. Effects of silibinin, EGCG, and their combination on the miRNA expression level of the miR-17−92 family in HUVECs cocultured with A549. Gene expression changes of miR-17 (A), miR-18a (B), miR-19b (C), miR-20a (D), and miR-92a (E) of HUVECs cocultured with A549 in response to EGCG (50 μg/mL), silibinin (50 μM), or the combination (50 μg/mL EGCG + 50 μM silibinin) were normalized to U6. Values represent mean ± SEM of at least two replicates. (Dissimilar letters indicate significant difference with max $P < 0.05$.)

Gene expression analysis indicated that EGCG, silibinin, and their combination significantly down-regulate miR-18a (Figure 5B), miR-20a (Figure 5D), and miR-92a (Figure 5E) in HUVEC cocultured with A549 cells. Concomitantly, silibinin downregulated miR-17 expression in HUVEC either in single treatment or in combination with EGCG. However, EGCG alone did not alter miR-17 in the same conditions (Figure 5A). On the other hand, miR-19b, as an anti-angiogenic factor, was significantly upregulated specifically after EGCG + silibinin co-treatment in HUVEC. Nevertheless, miR-19b did not significantly change upon EGCG or silibinin treatment compared to the control group (Figure 5C). Interestingly, the combination of EGCG and silibinin did not significantly change the expression of miR-17−92 cluster when HUVEC was cocultured with normal fibroblasts obtained from healthy subjects (data not shown).

Our data suggest that the expression of pro-angiogenic miRNAs in endothelial cells elevated in the presence of tumor cells could be modulated upon EGCG, silibinin, or combination treatment. More interestingly, the combination of silibinin and EGCG treatment in endothelial cells is required to provoke a miRNA-mediated anti-angiogenic response, which was abolished by tumor cells.

Differential impact of these compounds on pro- versus anti-angiogenic miRNAs along with their suppressive effect on the VEGF−VEGFR2 axis provides mechanistic evidence to support the notion that the combination of EGCG and silibinin could effectively minimize angiogenesis in solid tumors. Nevertheless, extensive in vivo pharmacokinetic and mechanistic studies on animal models of cancer will determine the potential future application of this combinatorial therapy in clinical settings. In addition, in silico modeling investigations will further reveal the mode of interaction between EGCG and silibinin and specific molecular targets on endothelial cells.

■ CONCLUSIONS

Targeting multiple pathways of tumor angiogenesis along with minimal side effects on normal tissues is a demanding factor in the development of anticancer therapeutic strategies. A variety of flavonoids exhibit anti-angiogenic effects by targeting a wide range of molecular targets in both tumor and endothelial cells.[9] Herein, we have addressed the inhibitory effect of the EGCG and silibinin combination on endothelial cell migration, survival, *VEGF−VEGFR2*, and miR-17−92 expression, as essential events in angiogenesis. Altogether, our results suggest that the EGCG and silibinin combination may not only beneficially modulate endothelial cell functions but also directly target tumor cells. It could further the anti-angiogenic antitumor properties by widening the target cells, which deserves detailed investigations in the future.

Anti-angiogenic therapy has extensive benefits, which is based on the critical reliance of solid tumors on neo-angiogenesis.[7b,56] However, there are a number of challenges in front that encourage researchers to develop more efficient and less toxic approaches.[6] High distribution of flavonoids in fruits and vegetables and widespread consumption of plant products containing a variety of flavonoids as food or beverage all over the world, in parallel with growing evidence of their antioxidant and anticancer capacity, have made them promising alternatives for anti-angiogenic therapies.[57]

In addition, a variety of in vivo studies and human clinical trials on whether oral administration or intravenous injection of some flavonoids introduces an inconsistency with in vitro results which specify a severe concern about the nonsoluble

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flavonoids in water and the stability of these compounds in physiological conditions.[57,58] This would affect not only the bioavailability of the flavonoid but also degradation by enzymatic reactions, starting from mixing with saliva, and is capable of forming pro-oxidant molecules with possible side effects.[59] As an example, in the case of EGCG, the peak plasma level of orally administered flavonoid is in sub-micromolar range,[60] which is very low compare to approved active concentrations in an in vitro situation. To enhance the exploitation of the compound, there are some solutions, among which increasing the intestinal absorption by a nano-drug delivery system using polymeric micelles has been reported to exhibit a variety of benefits.[61] Sustained drug release, increased drug load, enhanced tumor accumulation, and high stability[61,62] are among the welfares of using the polymeric micelle approach, and improved efficacy has been reported for a number of flavonoids including EGCG[63] and quercetin.[62] Low solubility of silibinin in water, however, can be overcome by increasing the administered doses because highly tolerable characteristics of its consumption have been approved in a variety of in vivo and clinical studies. Cumulative uptake amount of this flavonoid in parallel with introducing the novel silibinin formulation can intensify bioavailability and plasma absorption.[19] However, it is beneficial to be cautious about using silibinin in combination with other drugs. In a clinical study of using oral administration of a commercial formula of silibinin, silybin-phytosome, in prostate cancer patients, an improvement in the bioavailability and plasma absorbance of silibinin was observed; however, variability in inter- or intrapatient responses emphasizes the impact of complexity of physiological conditions on its functionality and necessitates wide and detailed preclinical studies prior to using flavonoids in clinical conditions. Altogether, these are suggestive of evaluating promising drug delivery approaches for future studies on EGCG + silibinin in in vivo and further clinical trials.

■ EXPERIMENTAL SECTION

Cell Culture. Human umbilical vascular endothelial cell (obtained from the Medical Biology Research Center of Kermanshah University of Medical Sciences) and the A549 cell line (ATCC CCL-185) (obtained from the Pasture Institute of Iran) were grown in Dulbecco's modified Eagle's medium (DMEM) (Bioidea) supplemented with 10% fetal bovine serum (FBS) (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Bioidea) in a humidified incubator at 37 °C and 5% CO_2.

Human Dermal Fibroblast Isolation. Human dermal fibroblasts (HDFs) were isolated from the obtained foreskin tissue samples of children (age range between 10 days and 2 months) immediately after circumcision by modifying and setting up the method reported by Nejaddehbashi et al.[64] Briefly, tissue samples obtained from a private clinic were transferred to the laboratory on ice-cold phosphate-buffered saline (PBS) containing penicillin (200 U/mL), streptomycin (200 μg/mL), and 0.3% amphotericin B. After sterilizing the samples with 70% ethanol and washing with PBS (200 U/mL penicillin, 200 μg/mL streptomycin, and 0.3% amphotericin B) three to five times, the hypodermis layer and related blood vessels were removed from the tissues. Samples were cut into 1 cm pieces and incubated in 0.25% trypsin−ethylenediaminetetraacetic acid (Bioidea) at 4 °C overnight. After incubation, the epidermis was set apart from the dermis, the dermis was chapped into very small pieces, and collagenase IV (1 mg/mL) was allowed to the tissue pieces for 1 h in an incubator at 37 °C, 5% CO_2, and 95% humidity, shaking every 5 min. After neutralizing with an FBS-containing medium, the suspension was centrifuged at 1600 rpm for 5 min and the supernatant was cultivated in cell culture flasks with 20% FBS.

Treatment Preparation. EGCG (Sigma-Aldrich, CAS number: 989-51-5, purity (high-performance liquid chromatography (HPLC) area %): 94%) and silibinin (Sigma-Aldrich, CAS number: 22888-70-6, purity (HPLC area %): 99.1%) were obtained from Sigma. EGCG and silibinin high-concentration stock solutions were prepared by dissolving the compounds in appropriate solvents, water for EGCG and dimethyl sulfoxide (DMSO) for silibinin. Treatment solutions were prepared freshly just before the experiment by diluting the appropriate amount of stock solutions in 1% FBS-containing medium. The final concentration of DMSO did not exceed 0.1% in culture medium.

Cell Viability Assay. Cell viability in response to different treatments was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded in a 96-well plate (0.5×10^4 cells/well) and treated with different concentrations of EGCG, silibinin, or their combination in DMEM containing 1% FBS. After 24 h, cell viability was compared with the control group using the MTT assay (Autocell) at 570 nm with the reference wavelength of 630 nm.

Wound Healing Assay. The migration capacity of different cells was evaluated via the wound healing assay. Briefly, cells were grown in 24-well plates at high density in DMEM containing 10% FBS and abovementioned antibiotics. Next day, a scratch was created across the confluent cell layer using a tip. After gently removing the old medium and detached cells, fresh medium supplemented with 2% FBS and different concentrations of treatments including EGCG (25 and 50 μg/mL), silibinin (50 and 75 μM), and their combination (25 μg/mL EGCG + 50 μM silibinin, 50 μg/mL EGCG + 50 μM silibinin, 25 μg/mL EGCG + 75 μM silibinin, 50 μg/mL EGCG + 75 μM silibinin) was added to each well. After 24 h incubation at 37 °C, 95% humidity, and 5% CO_2, the number of migrated cells was calculated in each treatment in four randomly chosen microscopic fields and compared with the nontreated control using ImageJ software.

Cell Coculture in Transwell Plates. Transwell plates (Corning, Cat# 3493) were used to evaluate the effect of different treatments on gene expression changes. After seeding and attachment of HUVECs in lower chambers, A549 cells were cultured in upper chambers of 12-well transwell plates and supplemented with DMEM, 10% FBS, and antibiotics. Coculture cells were treated with freshly prepared EGCG (50 μg/mL), silibinin (50 μM), or their combination (50 μg/mL EGCG + 50 μM silibinin) in 2% FBS for 24 h. The experiments were performed in triplicate and in two independent repeats.

RNA Extraction and RT-PCR. Total RNA of cocultured cells was extracted using RNX-plus (CinnaGen, Iran) according to the manufacturer's instructions. The yield and purity of the extracted RNAs were assessed by 2% agarose gel and NanoDrop 1000 (Termo Scientific), and complementary DNAs were synthesized using the PrimeScript RT reagent kit (Takara Bio, Japan) according to the manufacturer's protocol.

Quantitative Real-Time PCR. Gene expression changes of *VEGF*, *VEGFR*1, and *VEGFR*2 were evaluated in cocultured

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Table 2. Primer Sequences Used in RT-PCR

gene ID	name	strand	primers 5′−3′	product size	annealing T_m
7422	*VEGF*	forward	CTACCTCCACCATGCCAAGT	174	56
		reverse	CACACAGGATGGCTTGAAGA		
3791	*VEGFR2*	forward	GCGATTGAAAGAAGGAACTAGA	166	54
		reverse	TAGTCTTTGCCATCCTGCTG		
2597	*GAPDH*	forward	ACTCTGGTAAAGTGGATATTGTTGC	162	54
		reverse	GGAAGATGGTGATGGGATTTC		

HUVECs after different treatments using an Applied Biosystem StepOne instrument (Applied Biosystem) and SYBR Premix Ex Taq II (Takara Bio, Japan) according to the manufacturer's protocol. Quantitative real-time PCR conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the endogenous control to normalize changes of target genes through the $2^{-\Delta\Delta C_t}$ method. All samples were duplicated and repeated at least in two different biological repeats. Primer sequences are mentioned in Table 2.

Statistical Analysis. All data were obtained from at least two independent experiments and expressed as the mean ± SEM. One-way analysis of variance (ANOVA) with the Tukey post-hoc test was used to determine the effectiveness of different treatments compared with the control group. Two-way ANOVA with Tukey post-hoc analysis was utilized in assessment of the difference between treatment groups. The *P*-value less than 0.05 was considered as statistically significant.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00224.

Decreased cell viability of HUVEC, A549, and HDF in a dose-dependent manner in response to higher concentrations of EGCG and silibinin (PDF)

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Notes

The authors declare no competing financial interest.

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