International Journal of Phytomedicine 9 (2017) 511-517
http://www.arjournals.org/index.php/ijpm/index

Original Research Article

ISSN: 0975-0185

Aloe-emodin triggers ROS and Ca^{2+} production and decreases the levels of mitochondrial membrane potential of human brain capillary endothelial cells

Ivanka Dimova[1*], Svetla Danova[2], Ekaterina Nikolova[1] and Miglena Koprinarova[3]

***Corresponding author:**

Ivanka Dimova

[1]Department of Medical Genetics, Medical Faculty, Medical University of Sofia; address: 2 Zdrave Str., Sofia, Bulgaria, post code 1431.
[2]The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences; address: 26 Akad. G. Bontchev Str., Sofia, Bulgaria, post code 1113.
[3] The Roumen Tsanev Institute of Molecular Biology, 21 Akad. G. Bontchev Str., Bulgarian Academy of Sciences, Sofia, Bulgaria, post code 1113.

Received: 22 June 2017
Accepted: 04 August 2017
Published: 02 September 2017

Abstract

The aim of this work was to investigate the mechanisms of cytotoxicity of phyto-hydroxyanthraquinone aloe-emodin (AE) on human brain micro vascular endothelial cell line hCMEC/D3 and to assess the cellular response in the early stage of treatment in order to extend the knowledge of AE's anti-angiogenic properties. The immortalized human brain capillary endothelial cells hCMEC/D3 were treated with a series of AE concentrations (5 - 200 μM) for a period of 24 hours. The cell viability was determined by MTS assay. The cellular adenosine triphosphate (ATP) levels were evaluated by CellTiter-Glo® luminescent assay. The intracellular reactive oxygen species (ROS) were determined by 2',7'-dichlorofluorescein (CM-H2DCFDA) fluorescence assay. The mitochondrial membrane potential (MMP) was assessed using tetramethylrhodamine methyl ester (TMRM) staining, while Fluo-4 was used to measure the intracellular free Ca^{2+} concentrations inside living cells analysed by High Content Analysis using the Arrays can VTI 740.

Twenty-four-hour treatment of hCMEC/D3 cells with AE, in concentrations between 50 and 200 μM, decreased the cell viability as well as the intracellular ATP levels in a dose-dependent manner. Increased ROS production and disruption of the mitochondrial membrane potential have also been detected. Notably, AE at a concentration greater than 5 μM dramatically increased intracellular calcium levels.

Our results collectively indicate that AE inhibits proliferation of human brain micro vascular cells via a mechanism involving ROS generation, disruption of Ca^{2+} homeostasis and mitochondrial damage.

Keywords: aloe-emodin, human brain capillary endothelial cells, reactive oxygen species, mitochondria, intracellular Ca^{2+}

Introduction

The creation of new vascular network through stimulation *via* pro-angiogenic signals of the quiescent endothelial cells from the adjacent blood vessels to change their phenotype to highly proliferative, to migrate and finally to differentiate, forming new capillaries to the tumor mass, is essential for the primary tumor growth and its subsequent metastasis. Therefore, blocking angiogenesis is considered an effective strategy to stop the growth and metastasis of the tumor and extend the lives of patients.

Angiogenesis plays an important role in brain tumors growth and progression [1]. Anti-angiogenic therapies including bevacizumab are being routinely used in the treatment of patients with malignant glioma [2]. However, the use of bevacizumab leads to significant toxicity, including hypertension and proteinuria, thromboembolic events and poor wound healing [2]. New anti-angiogenic agents, with better efficacy of treatment and lower toxicity, are being researched and developed. Discovery and study of natural compounds with anti-angiogenic activity, capable of controlling the growth of blood vessels, inhibiting metastasis and inducing apoptosis in cancer cells are in particular interest [3,4]. Moreover, natural substances are characterized with low toxicity, low number of side effects and low cost [5]. Hundreds of natural compounds of plant and animal origin have properties of angiogenic inhibitors [6-8]. One of them is aloe-emodin [1,8-dihydroxy-3-hydroxymethylanthraquinone], bioactive hydroxyanthraquinone found in *Aloe barbadensis Miller (Aloe vera), Rheum officinale*, as well as in leaves and roots of other plants [9], showing *in vitro* and *in vivo* antitumor activity and anti-angiogenic properties [10,11]. There are studies on AE-induced apoptosis in different tumor cell lines, although the molecular mechanisms are not precisely known

DOI:10.5138/09750185.2138

[12]. Notably, studies validating the modulating role of AE in the regulation of angiogenesis and metastasis are just a few [10,12] and very little is known about the mechanisms *via* which AE inhibits the proliferation of endothelial cells.

AE can influence all key steps involved in the angiogenesis, including the local proliferation of endothelial cells, migration, invasion and finally differentiation and new vessels formation. Data showed that AE inhibits cell proliferation and *in vitro* cell migration/invasion and tube formation of human umbilical vein endothelial cells (HUVECs) [11]. The ability of AE treatment to inhibit the proliferation of primary bovine aortic endothelial cells (BAEC) have been also proven [10]. Other anthroquinone derivatives, rhein [7] and emodin [13], also possess anti-angiogenic activity, inhibiting vessel formation in zebra fish embryos, HUVEC proliferation, migration and tube formation as well as vessel formation in chick embryos and proliferation of BAEC cells, respectively.

It was shown that some anthraquinones and their derivatives induce apoptosis in tumor cells *via* increased ROS and Ca^{2+} production and disruption of mitochondrial membrane potential [14]. Recently, the antiproliferative effect of AE on human breast adenocarcinoma cell line MCF-7, associated with intracellular Ca^{2+} signaling, was found [15]. However, although data indicate that AE behaves both as an antitumor and an anti-angiogenic compound [10], from the best of our knowledge, there are no studies about related processes into endothelial cells, nor about the effect of AE on human brain capillary endothelial cells, in particular.

In order to get inside the molecular mechanisms by which AE affect endothelial cell proliferation, we studied its dose-dependent cytotoxic effect on brain micro vascular endothelial cells. For this purpose, using hCMEC/D3 cells (immortalized human brain capillary endothelial cells), we examined the effect of treatment with AE on the integrity of the plasma membrane, ROS production, mitochondrial membrane potential and intracellular calcium levels in the cytosol of the treated with different concentrations of AE cells.

Material and methods

Chemicals and reagents

Aloe-emodin, purchased from Apply Chem. was dissolved in dimethyl sulfoxide (DMSO) in a stock solution at a concentration of 25 mM and stored at -20°C until used. The stock of AE was dissolved with assay medium EBM-2 to a necessary final concentration prior to being used. The fluorescent dyes Hoechst 33342, Tetramethylrhodamine methyl ester (TMRM), ToPro-3 and Fluo-4 were obtained from Life Technologies.

Cell culture

The hCMEC/D3 cell line was kindly donated by Florence Miller, B.B. Wecksler (Inserm, France) to the group of Prof. Kenneth Dawson. The work with this cell line was carried out in University College Dublin (UCD). Immortalized brain capillary endothelial hCMEC/D3 cell line have been created from brain endothelial cells, isolated from human brain tissue via surgical excision of an area of the temporal lobe of an adult female with epilepsy [16]. The endothelial cells were immortalized by lentiviral transduction of the catalytic subunit of human telomerase and SV40-T antigen. The morphological characteristics and the expression of numerous typical endothelial markers and cell adhesion molecules were previously shown [16]. hCMEC/D3 cells were cultivated in a cell culture medium EBM-2 supplemented with vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IL-1), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), fetal calf serum (FCS), gentamicin sulfate/amphotericin B and hydrocortisone (Lonza Biosciences). The hCMEC/D3 cells were used from passage 7–20. For assays, cells were supplemented with growth factor depleted EBM-2 assay medium containing bFGF, 2% FCS, hydrocortisone and 10 mM HEPES during monolayer formation. The cells were cultivated in an incubator at 37˚C with 5% CO_2 and saturated humidity.

MTS assay

The cell viability was tested by MTS assay which measures the reduction of [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) to formazan by mitochondria in living cells. hCMEC/D3 cells in assay medium were seeded in 96-well plates at a density of 1 x 10^4 cells/well and cultivated for 4 days. MTS assay was carried out after 24-hour exposure of AE at concentrations of 0, 50, 100 and 200 µM. Dimethylsulfoxide (DMSO 0.8%) was used as a control. After 24 hours of exposure of AE, the original cell culture was replaced with 100 µl of fresh phenol red free medium and 20 µl of the MTS reagent was added directly to each well. The 96-well plates were incubated for 60 min at 37 C in a humidified atmosphere of 5% CO_2. Formazan absorption was measured at 490 nm using a micro titer plate reader (Varioskan Flash, Thermo Scientific, MA, USA).

ATP assay

Intracellular levels of adenosine triphosphate (ATP) were measured with the Cell Titer-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer´s recommendations. Briefly, 1 x 10^4 hCMEC/D3 cells in assay medium were plated onto 96 multi-well plate. The incubation was carried out for 4 days. Then, the cells were exposed to different concentrations of AE for 24 hours prior to ATP measurements. After incubation, an equal volume of the assay reagent was added to the cells and incubation was continued for 30 more minutes at room temperature. Luminescence was measured

using Flash plate reader (Thermo Fisher Scientific). Results were normalized against the untreated control.

High content analysis (HCA)

Cells ($1x10^4$) were plated in a 96 well plates in 150µl assay medium. After three days of incubation, the cells were treated with AE at concentrations of 0, 5, 25, 50, 100 and 200 µM for 24 hours. Then, 50 µl of medium was removed from the wells and replaced with 50 µl of the following reagents to reach the pointed final concentrations: Hoechst (0.4 µM), TMRM (20 nM), Fluo-4 (1 mM) and ToPro-3 (1 µM). After one-hour incubation, living cells were analyzed by High Content Analysis using the Arrays can VTI 740 (Thermo Scientific).

Measurement of ROS production

ROS production was measured using chloromethyl- H2DCFDA (Molecular Probes) according to the manufacturer's instructions. The samples were analyzed by flow cytometry with a CyAn ADP cytometer (DAKO), using the Summit software (DAKO).

Statistical analysis

Data analyses were performed by using *t*-tests for comparison between two groups (AE-treated cells from each of the groups with untreated controls). The level of statistical significance was set at $p < 0.05$. The results were presented as mean ± standard deviation (SD). All statistical analyses were performed using Excel 2013.

Results

Cytotoxic effect of AE on hCMEC/D3 cells

To determine the inhibitory activity of AE on brain capillary endothelial cells we used MTS assay. MTS assay was carried out after 24-hour exposure of AE in concentrations of up to 200 µM. The results showed that the treatment with AE in concentrations greater than 50 µM decreased the cell viability in the first 24 hours of exposure (Figure-1).

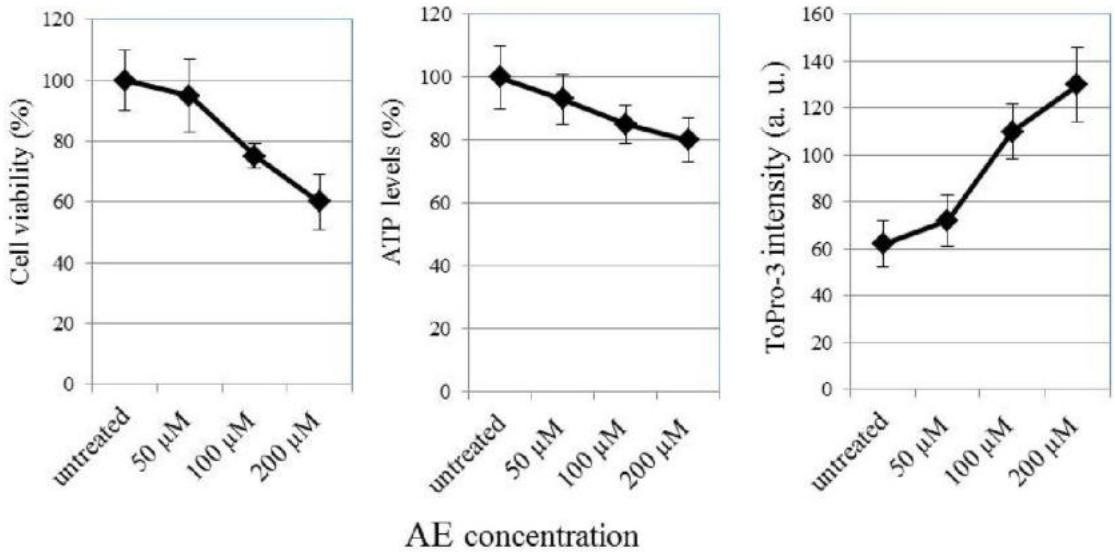

Figure-1. Effect of AE treatment on hCMEC/D3 cell viability. The cells were incubated with 0-200 µM AE for 24 hours. The ability of the cells to bioreduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium into a formazan product was analyzed by MTS assay. The assessment of ATP levels into the treated cells were detected by Cell Titer-Glo® Luminescent Cell Viability Assay. Metabolic activity measured by MTS and ATP assays was compared with ToPro-3 uptake, indicative of the plasma membrane integrity. The permeabilization of the plasma membrane was analyzed by staining the live cells with ToPro-3 and HCA. Results are expressed as the mean ± SD of three experiments. Triplicate wells were used for each study group. $p<0.05$ *versus* the untreated control group.

To confirm the results above we used DNA dye ToPro-3, which is useful as a nuclear counter stain and dead-cell indicator, since ToPro-3 did not penetrate intact cellular membranes. After 24-hour exposure of different concentrations of AE (0-200 µM) we stained the live cells with ToPro-3 and evaluated the cell viability by HCA. In a full agreement with MTS test, the results received from the HCA showed an increase in the dead cells in the populations after a twenty-four-hour exposure at 100 µM and especially at 200 µg/ml AE (Figure-1).

In order to assess the cytotoxic potential of AE on hCMEC/D3 cells, we also determined the cellular adenosine triphosphate (ATP) levels, having in mind that ATP degrades rapidly in dead cells and decreases in injured cells [17]. The endothelial cells were cultured in drug-free medium or in the presence of increasing AE

concentrations, as in the above-mentioned experiments. A dose-dependent decrease in viable cell numbers was observed, detectable at concentrations of 50 µM and above (Figure-1).

AE treatment induced increased ROS production in hCMEC/D3 cells

To examine intracellular reactive oxygen species formation we used CM-H2DCFDA (derivative of 2',7'-dichlorofluorescein) as a detection reagent. The flow cytometer was used to quantify the resulting fluorescence intensity using 488 nm excitation and measuring fluorescence emission at 530 nm. The hCMEC/D3 cells were treated with AE for 8 hours. As a positive control, we used amine-modified polystyrene nanoparticles in concentration 100 µg/ml, which have been previously proven to induce ROS formation in a human brain astrocytoma cell line [18]. The results showed that AE (10-50 µM) stimulated ROS formation in a concentration-dependent manner (Figure-2), compared with the untreated control.

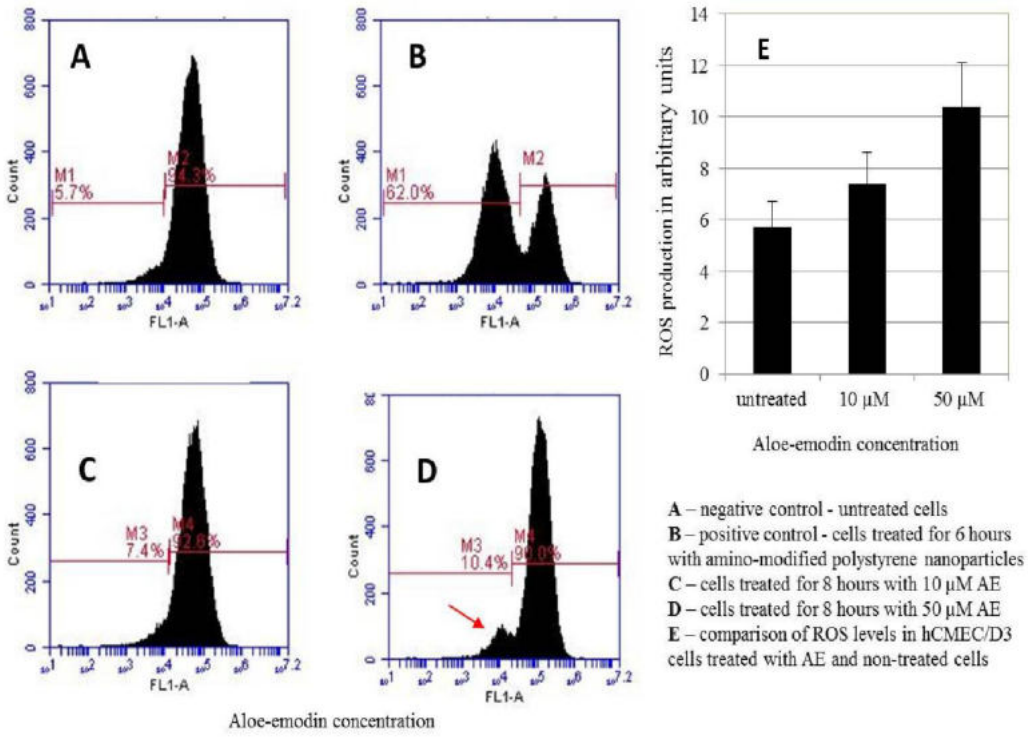

Figure-2. AE induces an increase in ROS levels in human brain endothelial cells. hCMEC cells were incubated with 0, 10 and 50 µM AE for 8 hours. Amine-modified polystyrene nanoparticles, in concentration 100 µg/ml, were used as a positive control. ROS generation were assessed *via* CM-H2DCFDA as a detection reagent. Results are expressed as the mean ± SD of three experiments. $p<0.05$ *versus* the untreated control group.

AE disrupted the mitochondrial membrane potential

We further investigated the correlation of ROS generation with MMP alterations following AE administration. MMP is a reliable indicator of mitochondrial function [19]. To analyze whether AE induces changes in mitochondria we used the live-cell permeant fluorescent indicator of mitochondrial membrane depolarization TMRM. We treated hCMEC/D3 cells for 24 hours with AE in concentrations from 50 µM to 200 µM and *via* HCA we detected dose-dependent decrease in mitochondrial membrane potential (Figure-3).

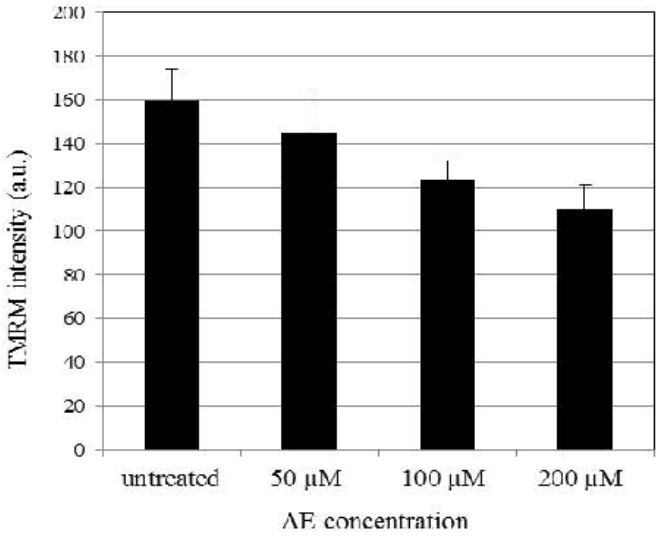

Figure-3. Effect of AE on the mitochondrial membrane. hCMEC/D3 cells were incubated with different concentrations of AE (0-200 µM) and mitochondrial membrane potential changes were analyzed by staining the live cells with TMRM for one hour. The changes in the intensity of TMRM fluorescence were followed by HCA. Results are expressed as the mean ± SD of three experiments. Four wells were used for each study group. $p<0.05$ *versus* the untreated control group.

AE increased intracellular calcium in a dose-dependent manner

The collapse of the MMP could result in disruption of calcium homeostasis. In order to decipher the molecular basis of toxicology mechanism induced by AE on the brain endothelial cell line, we investigated the intracellular free Ca^{2+} inside the living cells using calcium indicator Fluo-4 and HCA. Treatment with AE at concentrations 25 µM and higher dramatically increased intracellular Ca^{2+} levels (Figure-4). Even at 5 µM, the intracellular calcium level was elevated almost twice (over 85%), compared to untreated control, showing that AE significantly changes calcium homeostasis into hCMEC/D3.

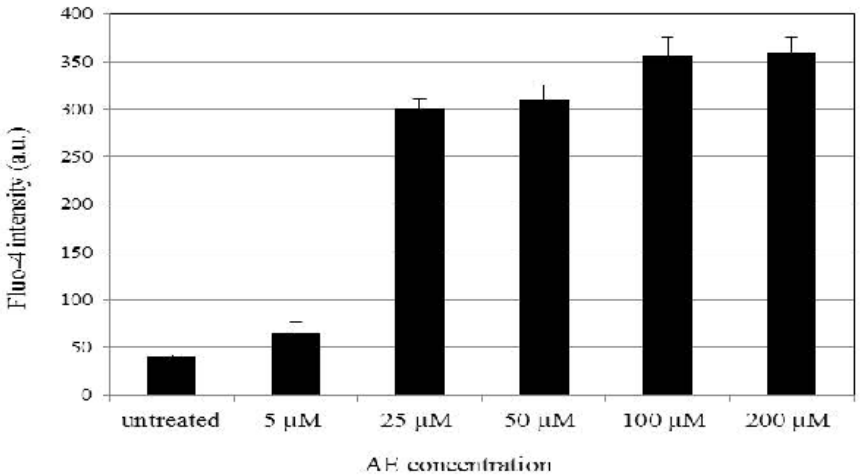

Figure-4. AE induced intracellular free calcium elevation in human brain micro vascular cells. The hCMEC/D3 cells were treated with AE (0-200 µM) for 24 hours. Fluo-4 and HCA were used to measure calcium concentrations inside living cells. Results are expressed as the mean ± SD of three experiments. Four wells were used for each study group. $p<0.05$ *versus* the untreated control group.

Discussion

Experimental data from the literature showed that AE possesses anticancer activity inducing cell cycle arrest and apoptosis in multiple tumor cells and having anti-angiogenic and anti-metastatic activity as well [12].

Local proliferation of endothelial cells is the first step in the angiogenic process. Our findings suggest a dose-dependent inhibition of cell proliferation in human micro vascular endothelial cells treated with AE. Incubation of the cells with aloe-emodin induces ROS generation in a dose dependent manner, showing the pro-oxidant properties of AE in endothelial hCMEC/D3 cells. Many pro-oxidant agents are able to open mitochondrial permeability transition pore (mPTP) and to promote cellular death [20]. Oxidants can increase the release of intracellular Ca^{2+} from the endoplasmic reticulum [21] since ROS at high levels can target ER-based calcium channels leading to the release of Ca^{2+}. The high levels of Ca^{2+} lead to opening of the mPTP in order for the cell to be protected from cytosolic Ca^{2+} overload [22]. Elevated ROS can lead to a sustained mPTP opening, resulting in the release of pro-apoptotic factors and cell death [22]. Notably, from our experience, treatment with only 5 µM AE was enough to increase significantly Ca^{2+} in the brain endothelial cells.

The intracellular calcium concentration is crucial to the regulation of cell death and ROS acts as an upstream regulator of the calcium level [23]. Mitochondria may respond to a Ca^{2+} signal through enhanced production of ROS. A study using human papilloma virus-immortalized keratinocytes has shown that elevation of intracellular Ca^{2+} due to exposure to irradiation has caused an overload of Ca^{2+} in the mitochondria, thus leading to a transient loss of membrane potential and production of ROS [24]. In hCMEC/D3 endothelial cells, AE increases intracellular Ca^{2+} concentrations and induces changes in mitochondrial membrane potential, as well as ROS production.

Conclusions

In conclusion, our present study, for the first time to best of our knowledge, demonstrates aloe-emodin-mediated cytotoxicity on human brain capillary endothelial cells (hCMEC/D3). We assessed changes in the cellular ATP levels, intracellular ROS, mitochondrial membrane potential and intracellular free Ca^{2+} in live micro vascular endothelial cells. The results received might contribute to the assessment of the anti-angiogenic properties of this natural compound and provide opportunity for future clinical application of AE-related drugs.

Acknowledgments

The authors thank prof. Kenneth Dawson, Director of the Centre for BioNano Interactions, University College Dublin - National University of Ireland, Dublin for giving the opportunity to use hCMEC/D3 cell line and the instrumental base of his laboratories. Special thanks to Dr. David Garry for the technical advice and expertise.

References

[1]. Jain RK. Angiogenesis in brain tumours. Nat. Rev. Neurosci. 2007;8:610-22.
[2]. Armstrong TS. Management of treatment-associated toxicites of anti-angiogenic therapy in patients with brain tumors. Neuro. Oncol. 2012;14:1203-14.
[3]. Ng KW. Anti-angiogenic and cytotoxicity studies of some medicinal plants. Planta Med. 2010;76:935-40.
[4]. Sagar SM. Natural health products that inhibit angiogenesis: a potential source for investigational new agents to treat cancer—Part 1. Curr. Oncol. 2006;13: 14–26.
[5]. Zhou W. Pharmacological potential action of Rhein and its diverse signal transduction: A Systematic Review. World Journal of Pharmaceutical Research. 2014;3:3599-3626.
[6]. Ribatti D. Endogenous inhibitors of angiogenesis: a historical review. Leuk Res. 2009;33: 638-44.
[7]. He ZH. Anti-angiogenic effect and mechanism of rhein from Rhizoma Rhei. Phytomedicine. 2011;18:470-8.
[8]. Rodriguez-Nieto S. Antiangiogenic activity of aeroplysinin-1, a brominated compound isolated from a marine sponge. FASEB J. 2002;16:261–263.
[9]. Thomson RH. Naturally occurring quinones. Academic Press Inc. New York Book. 1971;1–38.
[10]. Cárdenas C. Evaluation of the anti-angiogenic effect of aloe-emodin. Cell. Mol. Life. Sci. 2006;63:3083-9.
[11]. Suboj P. Aloe emodin inhibits colon cancer cell migration/angiogenesis by down regulating MMP-2/9, Rho B and VEGF via reduced DNA binding activity of NF-κB. Eur. J. Pharm. Sci. 2012;45: 581-91.
[12]. Chen R. Potential antineoplastic effects of Aloe-emodin: a comprehensive review. Am J Chin Med. 2014;42:275-88.
[13]. Wang XH. Inhibitory effects of emodin on angiogenesis. Yao Xue Xue Bao. 2004; 39:254-8
[14]. Lin SY. Emodin induces apoptosis of human tongue squamous cancer SCC-4 cells through reactive oxygen species and mitochondria-dependent pathways. Anticancer Res. 2009; 29:327-35.
[15]. Amin IM. Involvement of Intracellular Calcium and MAPK in Aloe Emodin Induced Anti-Proliferative in MCF-7. ASM Science Journal. 2014;8:165–173.
[16]. Weksler BB. Blood-brain barrier-specific properties of a human adult brain

endothelial cell line. FASEB J. 2005;19:1872–1874.
[17]. Wang P. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PLoS One. 2010;5:10202.
[18]. Bexiga MG. Cationic nanoparticles induce caspase 3-7- and 9-mediated cytotoxicity in a human astrocytoma cell line. Nanotoxicology. 2011;5:557-67.
[19]. Bagkos G. A new model for mitochondrial membrane potential production and storage. Med Hypotheses. 2014;83:175-81.
[20]. Rasola A, Bernardi P. Reprint of "The mitochondrial permeability transition pore and its adaptive responses in tumor cells". Cell Calcium. 2015;56:437–445.
[21]. Camello-Almaraz C. Mitochondrial reactive oxygen species and Ca^{2+} signalling. Am. J. Physiol. Cell Physiol. 2006; 291:1082–1088.
[22]. Görlach A. Calcium and ROS: A mutual interplay. Redox Biol. 2015;6:260-71.
[23]. Huang FJ. Characterization of apoptosis induced by emodin and related regulatory mechanisms in human neuroblastoma cells. Int J Mol Sci. 2013;14:20139-56.
[24]. Lyng FM. The involvement of calcium and MAP kinase signalling pathways in the production of radiation-induced bystander effects. Radiat Res. 2006; 165:400-9.