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Original Article

Parietin as an efficient and promising anti-angiogenic and apoptotic small-molecule from *Xanthoria parietina*

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ABSTRACT

Lichens that are exclusive symbiotic organisms composed of fungus and alga, are considered as a wealthy source of biologically and pharmacologically active small-molecules thanks to the tight metabolic relationship between symbiotic partners. We herein report cytotoxic, anti-angiogenic and apoptotic profile of a lichen derived small-molecule named parietin. Parietin was isolated from the acetone extract of *Xanthoria parietina* (L.) Th.Fr (1860), Teloschistaceae, which was gathered from Afyon, Turkey. AlamarBlue™ cell viability, lactate dehydrogenase membrane leakage and PicoGreen™ dsDNA quantitation assays were used to determine the cytotoxic concentrations of parietin on cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma (HCC1428), human breast ductal carcinoma (T-47D), and human umbilical vein endothelial (HUVEC) cells. Additionally, cell adhesion, endothelial tube formation, reactive oxygen species accumulation and active caspase 3 determination assays were employed to identify the anti-angiogenic and apoptotic efficiency of parietin. Low concentrations of parietin such as 50 and 100 μM showed a significant anti-angiogenic and apoptotic activity though the half-maximal inhibitory concentration (IC_{50}) values were higher than 600 μM on the cells. On the other hand, it was observed that parietin shows less cytotoxic and membrane degradative activities on healthy HUVEC cells than the HCC1428 and T-47D breast cancer cells. Parietin seems to be a promising anti-angiogenic and apoptotic lichen metabolite for the further investigations.

Introduction

Breast cancer, the most common type of women's cancers, is considered as the major cause of cancer-related deaths worldwide though the surgical intervention in combination with radiotherapy is a beneficial treatment modality for the patients who have early stage breast cancer (Yang et al., 2018). It is apparently seen that there is an urgent need to explore and design novel treatment strategies and drugs to cure the late stage breast cancers (Gradishar, 2016). The ethno-pharmacological surveys indicate that the anticancer agents derived from natural sources are acknowledged and preferred as more effective and safer drugs by patients and physicians because there is a general consensus that synthetic chemotherapeutics might cause damages in healthy cells and tissues while they destroy the targeted cancer cells or arrest the progression of tumor tissues (Alasmary et al., 2017).

Although cisplatin is currently employed as the most common chemotherapeutic to treat various solid tumors such as the head and neck, lung, testicular, ovarian and breast cancers, the development of cisplatin resistance in human cancer cells is a major problem in the treatment of many patients and appears as a pervasive limitation in chemotherapy (Zheng et al., 2017). Zheng and coworkers (2017) hypothesized a molecular mechanism related to the development of cisplatin resistance and the intracellular level of 6-phosphogluconate dehydrogenase (6PGD) that is an oxidative pentose phosphate pathway enzyme, and they found that cisplatin-resistant human ovarian (C13*) and lung (A549DDP) cancer cells have higher levels of 6PGD compared to their cisplatin-sensitive counterparts (OV2008 and A549) (Zheng et al., 2017). On the other hand, parietin (**1**, CID 10639) is a natural anthraquinone derivative, which can be found in medicinal plant rhubarb, the marine-derived fungus *Microsporum* sp. and lichens, *e.g. Xanthoria parietina*, *Teloschistes flavicans*, can play an inhibitory role in the oxidative pentose phosphate pathway by targeting the 6PGD and glucose-6-phosphate dehydrogenase (G6PD) enzymes (Lin et al., 2015; Lopez-Tobar et al., 2016; Pan et al., 2018). This preliminary study reveals, for the first time, the anti-angiogenic

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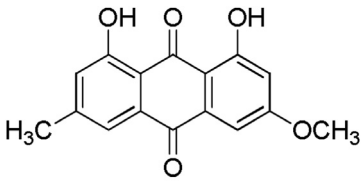
efficiency of lichen-derived parietin on human umbilical vein endothelial (HUVEC) cells, and itsanti-breast cancer and apoptotic influences on cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma (HCC1428) and human breast ductal carcinoma (T-47D) cells.



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Methods

Lichen material and isolation of the substance

Xanthoria parietina (L.) Th.Fr (1860), Teloschistaceae, was collected from *Quercus* sp. population located at 1.111 m above sea level in the northwestern part of Şeyhyahşi Village, Sandıklı, Afyon province, Turkey (N 38°27′03″, E 30°00′57″). A voucher specimen was stored in the Herbarium of Anadolu University (ANES), Turkey. Isolation of parietin (**1**, CID 10639) from the acetone extract of lichen and chemical characterization were actualized as previously described (Varol et al., 2015, 2016). Stock solutions of parietin were initially prepared in dimethyl sulfoxide (DMSO) at 0.05–0.1 M concentration and further diluted with fresh complete medium.

Cell culture condition

Cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma (HCC1428; CRL-2327™), human breast ductal carcinoma (T-47D; HTB-133™) and human umbilical vein endothelial (HUVEC; CRL-1730™) cells were purchased from American Type Culture Collection (ATCC) and maintained by following the culture method instructions of ATCC.

AlamarBlue™ cell viability assay

AlamarBlue™ cell viability reagent (DAL1025; Invitrogen Corporation, CA, USA) was used to determine the growth inhibitory activity of parietinas previously described (Hamid et al., 2004; Varol, 2018). Briefly, HCC1428 (15×10^3 cells/well), T-47D (15×10^3 cells/well) and HUVEC (10×10^3 cells/well) cells were plated in 96 well-plates and incubated for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. After the incubation period, the cells were treated by 25, 50, 100, 200 and 400 μM concentrations of parietin for 24 and 48 h. After incubation periods, AlamarBlue™ solution (10 ml/well) was added to the cells and incubated for further 4 h. To monitor the produced fluorescence, SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA) was employed at 560 nm excitation wavelength and 590 nm emission wavelength.

Lactate dehydrogenase (LDH) membrane leakage assay

Cytotoxicity Detection KitPLUS (04744926001; Roche Diagnostics GmbH, Mannheim, Germany) was used to determine the membrane degradation activity of parietin by monitoring the LDH leakage as previously described (Varol, 2018; Varol et al., 2016). The cells were seeded and treated as indicated in the AlamarBlue™ cell viability assay. ELX808IU microplate reader (Winooski, VT, USA) was employed to measure the absorbance at 490 nm.

PicoGreen™ dsDNA quantitation assay

Quant-iT™ PicoGreen™ dsDNA Assay Kit (P11496; Molecular Probes Inc., OR, USA) was used by following the manufacturer's instructions to determine the double-stranded DNA (dsDNA) quantitation of the cells that were plated and treated as described in the AlamarBlue™ cell viability assay. Fluorescence was measured using an excitation wavelength of 480 nm and emission wavelength of 520 nm in SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA).

Reactive oxygen species (ROS) accumulation assay

OxiSelect™ intracellular ROS assay kit (STA-342; Cell Biolabs Inc., CA, USA) was employed by following the manufacturer's instructions to determine parietin-mediated ROS accumulation into the cells that were plated and treated as described in the AlamarBlue™ cell viability assay. Fluorescence was analyzed by SpectraMax® M Series Multi-Mode Microplate Reader (Molecular Devices, LLC., Sunnyvale, CA) using excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Caspase 3 apoptosis determination assay

Colorimetric caspase 3 assay kit (CASP3C; Sigma-Aldrich Inc., MO, USA) was used by following the manufacturer's instructions to determine parietin-mediated caspase 3 activation into the cells that were plated and treated as described in the AlamarBlue™ cell viability assay. ELX808IU microplate reader (Winooski, VT, USA) was employed to measure absorbance at 490 nm.

Cell adhesion assay

Thiazolyl Blue Tetrazolium Bromide (M5655; Sigma–Aldrich Inc., MO, USA) was used to determine the anti-adherent activities of 25, 50 and 100 μM concentrations of parietin on HCC1428 (6×10^4 cells/well), T-47D (6×10^4 cells/well) and HUVEC (4×10^4 cells/well) cells as previously described (Humphries, 2001; Chen, 2011). Briefly, the cells were left at serum starvation for 8 h before the experiment. The cells were plated into 96-well plates and immediately treated with the sub-cytotoxic concentrations of parietin. After 30 min incubation at 37 °C in a humidified atmosphere containing 5% CO_2, the cells were washed three times with Phosphate-Buffered Saline (00-3002; Invitrogen Corporation, CA, USA) solution to remove non-adherent cells. The adherent cells were incubated for further 4 h in the serum-containing medium to allow the cells to maintain normal metabolic functions. After the incubation period, the adherent cells were measured by MTT assay as previously described (Varol et al., 2015, 2016). Absorbance was measured at 570 nm by ELX808IU microplate reader (Winooski, VT, USA).

Matrigel tube formation assay

Endothelial tube formation assay was performed as previously described (Cagir et al., 2017; Varol, 2018). Briefly, serum starved HUVEC cells (4×10^4 cells/well) were plated on the matrigel-coated wells in 96-well culture plates, and were equilibrated with EBM-2 (CC-3156; Lonza Group Ltd., Basel, Switzerland) medium containing the sub-cytotoxic concentrations of parietin. Endothelial tube formations were observed under an Olympus IX71 inverted microscope and photographed at 12, 24 and 48 h with an Olympus DP71 camera at 10x magnification. Angiogenesis Analyzer tool in

ImageJ 1.50i was employed to evaluate the microscope photos of endothelial tube formation assay. The parameters such as number of junctions, total meshes area, number of segments, total branching length and total segment length were considered to evaluate the anti-angiogenic activity of sub-cytotoxic concentrations of parietin.

Statistical data analysis

The experimental groups were expressed as percentage of the corresponding controls and compared using one-way ANOVA (Analysis of Variance) followed by the Tukey test in SPSS (Statistical Package for Social Science) software. Results were expressed as the mean ± standard deviation (SD), and asterisks indicate significant differences from the control groups by the Tukey test ($p < 0.05$). The half-maximal inhibitory concentration (IC_{50}) values were calculated by using nonlinear regression analysis of at least three separate triplicate experiments by "GraphPad Prizm 5" software. The figures were created by using Microsoft excel software and Photoshop CS6 was performed to organize the photos and figures.

Results

Proliferation and membrane integrity

Anti-proliferative and membrane degradative activities of parietin (**1**) were investigated by using AlamarBlue™ cell viability, lactate dehydrogenase (LDH) membrane leakage and PicoGreen™ dsDNA quantitation assays on HCC1428 and T-47D breast cancer, and healthy HUVEC endothelial cells (Fig. 1 and Table 1). The applied experiments showed that parietin hascytotoxic and membrane degradative effects in a concentration- and time- dependent manner though the calculated IC_{50} values were found higher than 600 μM for HUVEC, HCC1428, and T-47 D cells (Table 1). However, it was found interesting that parietin showed more cytotoxic and membrane degradative activities on the cisplatin-resistant BRCA2-mutated human breast TNM stage IV adenocarcinoma (HCC1428) than T-47D and HUVEC cells. On the other hand, it should be indicated that the obtained results from the proliferation and membrane integrity assays were compatible with each other, as can be seen in Fig. 1 and Table 1.

ROS accumulation and apoptosis

As can be seen in Fig. 2, HUVEC,HCC1428 and T-47D cells were treated by the sub-cytotoxic concentrations such as 25, 50 and 100 μM of parietin to determine the effect of parietin on ROS accumulation and caspase 3 activation (Fig. 2).The obtained results were compatible with the data of proliferation and membrane integrity assays (Fig. 1). It is explicitly observed that the sub-cytotoxic concentrations of parietinlead time- and concentration-dependent ROS accumulation that is known as an intracellular triggering factor of apoptosis, and induce concentration-dependent activation of caspase 3 that is an important indicator of apoptosis (Fig. 2).

Adhesion and angiogenesis

Endothelial tube formation assay and adhesion assay were performed to determine the anti-angiogenic and anti-adherent activities of parietin, respectively (Fig. 3, Supplement Figs. 1 and 4). As can be clearly seen in the Fig. 3, the sub-cytotoxic concentrations of parietin showed a significant anti-angiogenic activity by inhibition of the migration and tube formation of endothelial cells, and concentration dependent decreases were clearly observed in number of junctions, total meshes area, number of segments, total branching length and total segment length of endothelial tubes. However, the derived data from the adhesion assays showed that parietin has not a significant effect on the adhesion capabilities of endothelial cells (Fig. 4).

Discussion

Screening natural substances is considered as one of the most rational drug discovery strategies to find a convenient anticancer agent due to the insecure view of synthetic drugs and confidence in the traditional medicine knowledge (Bačkorová et al., 2012). Parietin (**1**, CID 10639), as a natural anthraquinone, exerts many biological and pharmacological activities including but not limited to the antineoplastic, apoptotic, anti-inflammatory, hepatoprotective, photoprotective, laxative and antimicrobial activities (Comini et al., 2017; Pan et al., 2018). In this study, the anti-breast cancer, anti-angiogenic and apoptotic activities of parietin were investigated on healthy HUVEC endothelial cells, cisplatin-resistant HCC1428 and T-47D breast ductal carcinoma cells.

In accord with the two different studies by Backorová and coworkers in 2011 and 2012, the applied proliferation and membrane integrity assays in this study displayed that parietin has a weak cytotoxic potential (Bačkorová et al., 2011; 2012; Panigrahi et al., 2018). Backorová and coworkers investigated the antiproliferative activity of parietin on the nine human cancer cell lines (A2780, HCT-116 p53+/+, HCT-116 p53−/−, HeLa, HL-60, HT-29, MCF-7, Jurkat and SK-BR-3) (Bačkorová et al., 2011; 2012). They hypothesized that parietin may be regarded as a cytostatic agent and might have target specific action due to the differences of action against various cancer cell lines. Moreover, it is worth noticing that the cytotoxic capability of parietin is far less than (-)-usnic acid and vulpinic acid that have been identified as anti-angiogenic lichen compounds (Koparal, 2015). Although the half-maximal inhibitory concentration (IC_{50}) of parietin for 48 h treatment on HUVEC cells was 1169.83 + 11.34 μM, IC_{50} values of (-)-usnic acid and vulpinic acid were reported 71.5 ± 0.19 μM and 231.94 ± 25.4 μM, respectively (Koparal, 2015). However, it should be noted that the convenient anti-angiogenic agents are expected to have low cytotoxic feature among with strong anti-migratory, anti-adherent and/or VEGF (vascular endothelial growth factor) inhibitory activities (Kadri et al., 2014; Varol, 2017). It is apparently identified that the increased levels of the oxidative pentose phosphate pathway enzymes is related to the cisplatin resistance and parietin has an inhibition ability on 6PGD and G6PD enzymes, we therefore thought that parietin is an appropriate compound to be employed as an adjunctive therapeutic agent though it has a weak cytotoxic activity (Pan et al., 2016; Zheng et al., 2017). On the other hand, extracellular matrix metalloproteinase inducer (EMPRIN), specificity protein 1 (Sp1), homeobox A5 (HOXA5), the AMP-activated protein kinase (AMPK)/Hypoxia-inducible factor 1α (HIF-1α) signaling pathway and C-X-C motif chemokine 12 (CXCL12)/C-X-C chemokine receptor type 4 (CXCR-4) signaling, have been evaluated as the probable targets of parietin (Chen et al., 2015; Liu et al., 2016; Pang et al., 2016; Gao et al., 2017; Pan et al., 2018).

Intracellular ROS accumulation and caspase 3 activation activities of parietin were investigated by using commercial assay kits because the previously published studies have indicated that parietin stimulates apoptosis through ROS/AMPK-dependent mitochondrial pathway (Xiong et al., 2015). In accord with the previously published studies, the obtained results showed that parietin provoked the intracellular ROS generation and accumulation and caspase 3 activation (Xiong et al., 2015; Pang et al., 2016). Thus, it can be hypothesized that parietin might promote apoptosis, cell cycle blockade and autophagy by modulating the ROS-related pathways and molecules such as AMPK/HIF-1α, DNMT1 or mitochondrial/apoptotic pathways, and EMMPRIN, SP1 or HOXA5 molecules (Chen et al., 2015; Xiong et al., 2015; Gao et al.,

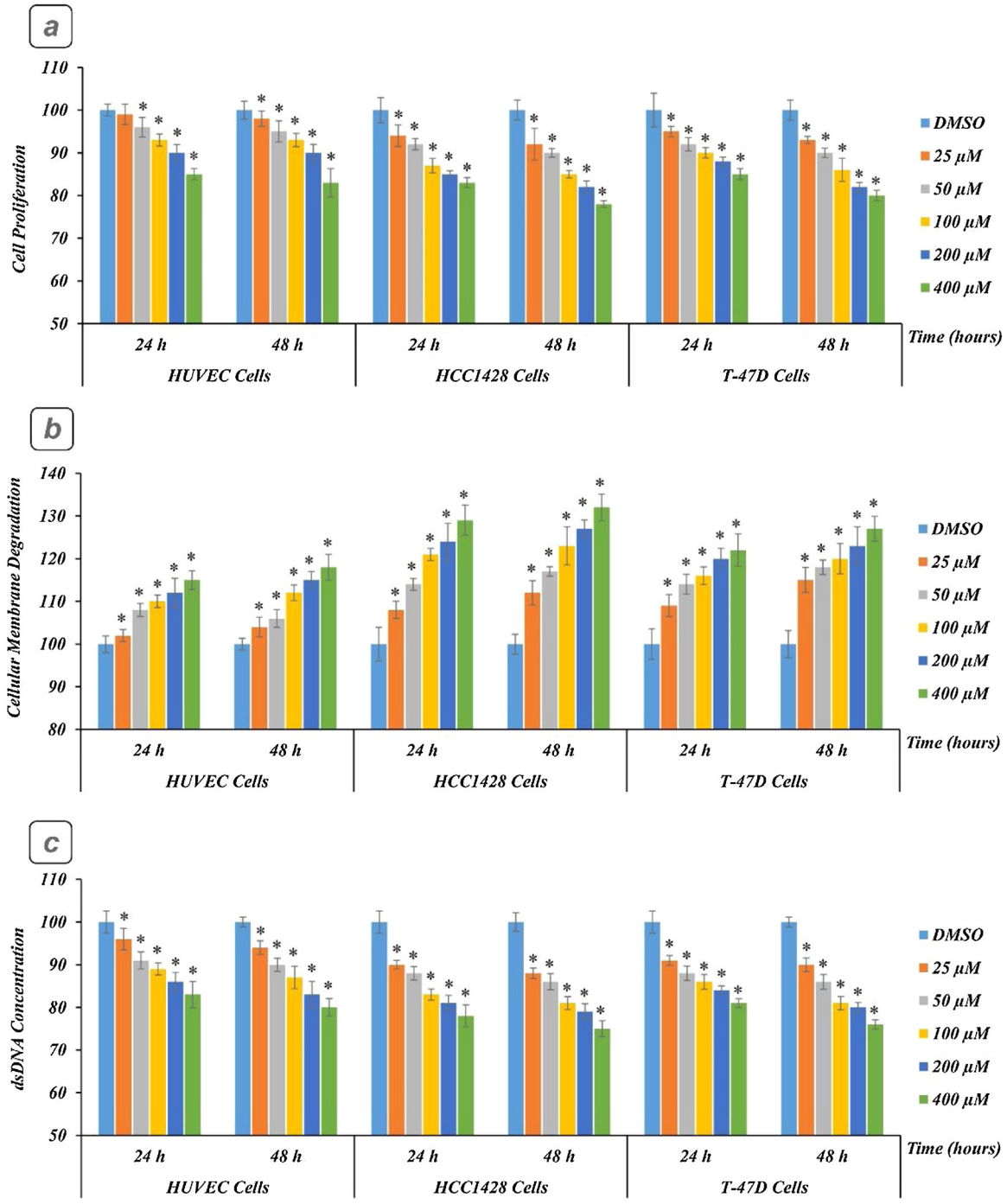

Fig. 1. Antiproliferative activity of parietin on HUVEC, HCC1428 and T-47D cells. (a) AlamarBlue™ cell viability assay, (b) lactate dehydrogenase (LDH) cellular membrane degradation assay, (c) PicoGreen™ dsDNA quantitation assay. Data were shown as mean ± standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$).

Table 1
IC_{50} values (µM) of parietin on HUVEC, HCC1428 and T-47D cells were calculated using nonlinear regression analysis in GraphPad Prizm 5 software (+SD; n = 3).

Toxicity assays	IC_{50} values on HUVEC		IC_{50} values on HCC1428		IC_{50} values on T-47D	
	24 h	48 h	24 h	48 h	24 h	48 h
Alamar Blue cell viability assay	1245.19 + 12.08	1169.83 + 11.34	927.05 + 8.96	762.62 + 7.35	1151.18 + 11.14	794.71 + 7.66
LDH cellular membrane degradation assay	1165.38 + 11.42	1024.48 + 10.04	664.65 + 6.53	628.06 + 6.17	825.31 + 8.11	692.88 + 6.81
PicoGreen dsDNA quantitation assay	980.58 + 9.48	831.31 + 8.02	696.43 + 6.71	610.55 + 5.86	819.05 + 7.89	627.27 + 6.03

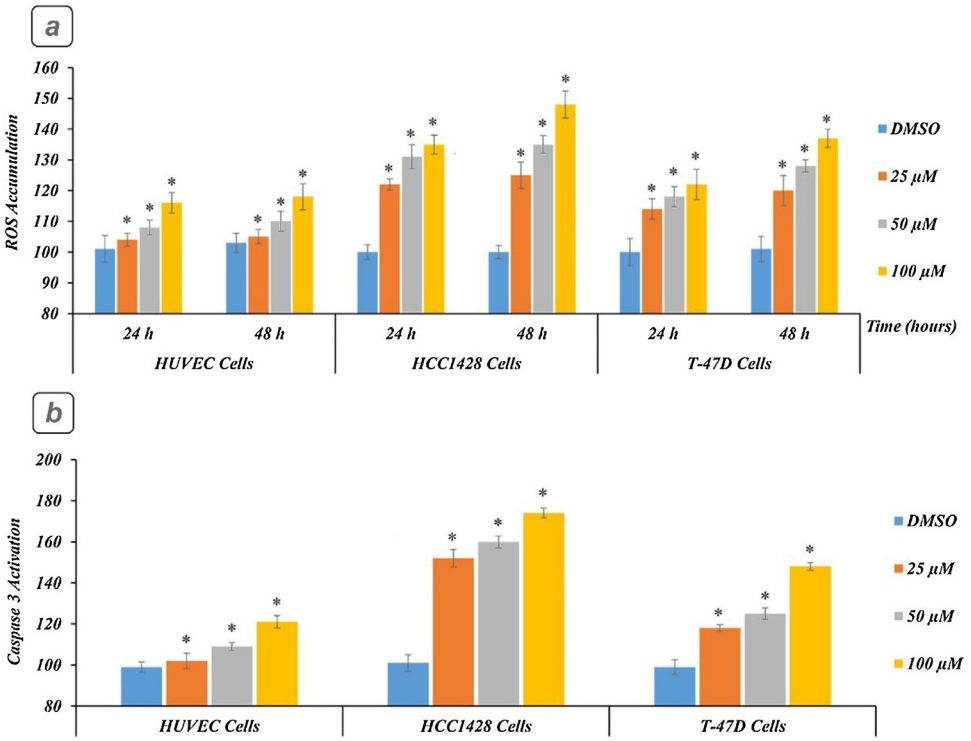

Fig. 2. Intracellular reactive oxygen species (ROS) accumulation (a) and Caspase 3 activation (b) activity of sub-cytotoxic concentrations of parietin.Data were shown as mean ± standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$).

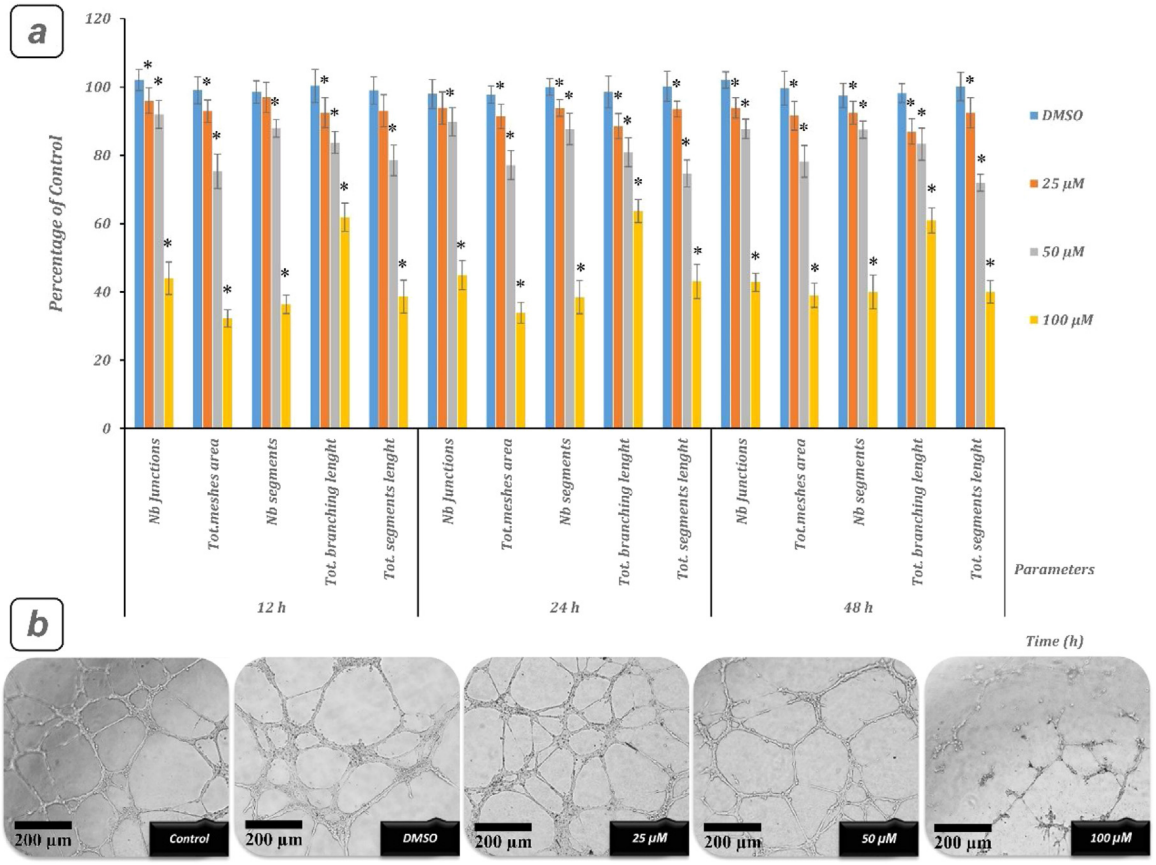

Fig. 3. Anti-angiogenic activity of sub-cytotoxic concentrations of parietin. (a) Histogram represents quantification of number of junctions, total meshes area, number of segments, total branching length and total segment length in the tube formation assay. Data were shown as mean ± standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$). (b) Representative images of HUVEC tube formation are shown for 12 h time point (10× magnification, scale bar: 200 µm).

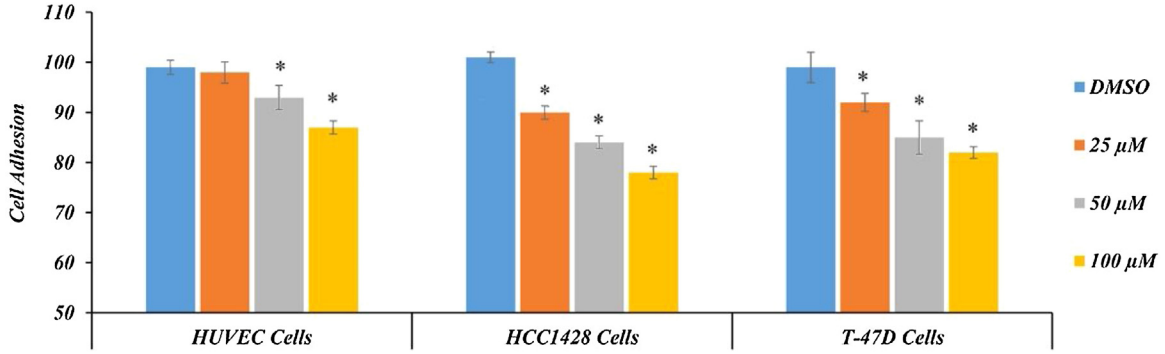

Fig. 4. Anti-adherent activity of sub-cytotoxic concentrations of parietin.Data were shown as mean ± standard deviation and asterisks indicate significant difference from the control group by the Tukey test ($p < 0.05$).

2017; Pan et al., 2016; Pang et al., 2016). However, it should be noted that there are some studies indicate that parietin did not cause significant changes of Annexin V positive cells, caspase 3 activation and cell cycle markers such as retinoblastoma protein, cyclin A, cyclin D1, P21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bačkorová et al., 2011, 2012; Basile et al., 2015). In these studies, the apoptotic ability of parietin has been considered as pretty limited though the current findings indicate that this conclusion is not completely correct when the apoptotic capacity of parietin is evaluated on its own rather than the comparison with the other lichen compounds such as usnic acid, atranorin and gyrophoric acid that are higher cytotoxic nature than parietin (Bačkorová et al., 2011, 2012).

On the other hand, it is well known that EMMPRIN stimulates tumor angiogenesis by elevating VEGF and matrix metalloproteinases (MMP) (Tang et al., 2005; Varol, 2017). To the best of our knowledge, the current study reveals the anti-angiogenic and anti-adherent efficiency of parietinfor the first time. According to the derived results from the applied endothelial tube formation and adhesion assays, parietin has a significant anti-angiogenic activity on human umbilical vein endothelial (HUVEC) cells though it has anti-adherent activity at a weak but distinguishable level. To inhibit endothelial tube formation, the required concentration of thalidomide, a commercial angiogenesis inhibitor, should be as high as 300 μM though 100 μM concentration of parietin completely inhibits endothelial tube formation (Koparal, 2015). The anti-angiogenic activity of parietin seems to be therefore promising and additionally related to its ability to inhibit EMMPRIN (Chen et al., 2015). Additionally, the current findings revealed that parietin has more anti-adherent activity on HCC1428 and T-47D breast cancer cells than on healthy endothelial cells though it has been precisely indicated by the immunohistochemical analyses in the literature that the expression level of focal adhesion kinase that is the primary regulator of the integrin-mediated focal adhesion assembly is higher in breast cancer cells than healthy cells (Cance et al., 2000). However, it should be noted that ROS production alters the organization of cytoskeleton and decreases the distribution of focal adhesion kinase in focal contacts(Mahdi et al., 2000). Therefore, the higher anti-adherent activity of parietin on breast cancer cells is considered to be associated with the higher ability to produce ROS in HCC1428 and T-47D breast cancer cells.

This study consequently reveals the cytotoxic, apoptotic and anti-angiogenic activity of parietin on human healthy endothelial (HUVEC) cell, cisplatin-resistant human breast adenocarcinoma (HCC1428) and human breast ductal carcinoma (T-47D). It could be plainly viewed that parietin has a great potential to be employed as an anti-angiogenic and adjunctive therapeutic agent thanks to its modulating ability of cellular pathways and/or molecular targets. The extensive *in vitro* and *in vivo* studies should be performed to understand the underlying activity mechanisms of parietin.

Authors' contributions

The author, who has the complete responsibility of the study and the manuscript, read and approved the final manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.04.012.

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