

## Role of peroxisome proliferator-activated receptor alpha and gamma in antiangiogenic effect of pomegranate peel extract

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### ARTICLE INFO

#### Article type:

Short communication

#### Article history:

Received: Feb 19, 2015

Accepted: Jun 18, 2015

#### Keywords:

Angiogenesis  
Peroxisome proliferator –  
activated receptors –  
(PPARs)  
Pomegranate  
Vascularendothelial –  
growth factor

### ABSTRACT

**Objective(s):** Herbal medicines are promising cancer preventive candidates. It has been shown that *Punica granatum* L. could inhibit angiogenesis and tumor invasion. In this study, we investigated whether the anti-angiogenic effect of pomegranate peel extract (PPE) is partly attributable to Peroxisome proliferator-activated receptors (PPARs) activation in the Human Umbilical Vein Endothelial Cells (HUVECs).

**Materials and Methods:** Ethanol extract from PPE was prepared. HUVECs were treated in four groups (with PPE (10 µg/ml) alone, PPE with or without PPAR $\gamma$  (T0070907) and  $\alpha$  (GW6471) antagonists, and control group). The possible effect of PPARs on angiogenic regulation was checked by Matrigel assay. The mRNA expression levels of vascular endothelial growth factor (VEGF) was detected by Quantitative reverse transcription-polymerase chain reaction (QRT-PCR).

**Results:** PPE significantly inhibited both tube formation (size, length, and junction of tubes) and VEGF mRNA expression ( $P < 0.05$ ). Our results showed that the anti-angiogenic effects of PPE were significantly reversed by both PPAR antagonists ( $P < 0.05$ ). There was no difference between PPE plus antagonists groups and the control group.

**Conclusion:** In summary our results showed that the anti-angiogenic effects of PPE could be mediated in part through PPAR dependent pathway.

#### ► Please cite this article as:

Dana N, Haghjooy Javanmard Sh, Rafiee L. Role of peroxisome proliferator-activated receptor alpha and gamma in antiangiogenic effect of pomegranate peel extract. Iran J Basic Med Sci 2016; 19:106-110.

### Introduction

Angiogenesis is the process of new blood vessel generation and is involved in the pathophysiology of a wide variety of diseases such as cancer (1, 2). Anti-angiogenic therapy represents a new approach to the early intervention and prevention of malignant disease (3). It is highly desirable to find dietary sources of anti-angiogenic molecules that have the ability to reduce cancer risk as well.

*Punica granatum* L. (pomegranate) has been known for its several beneficial effects including prevention and treatment of several types of cancer through its various metabolites (4).

Pomegranate peels are characterized by large amounts of phenolic compounds, and their great antioxidant potential (5). We previously showed that pomegranate peel extract (PPE) inhibits Human Umbilical Vein Endothelial Cells (HUVECs) angiogenesis, VEGF mRNA expression and secretion (6).

Previous studies have shown that PPE inhibited cell proliferation and angiogenesis marker expression (7, 8).

Recent research demonstrated that some parts of pomegranate inhibit angiogenesis via reduction of vascular endothelial growth factor (9). Also experimental studies have shown that the skin and arils of pomegranate extract inhibit tumor angiogenesis (10).

Although previous studies have proved the effectiveness of anti-angiogenic effect of pomegranate compartments but still the mechanisms underlying its anti-angiogenic activity and specially PPE remain unknown.

Some studies have shown that pomegranate carries out its therapeutic effects by affecting the Peroxisome proliferator-activated receptors (PPARs) pathway (11, 12).

PPARs are ligand-activated transcription factors that perform diverse metabolic functions (13). Three subtypes of PPARs ( $\alpha$ ,  $\beta$  or  $\delta$ ,  $\gamma$ ) have been recognized, each encoded by distinct genes and expressing in a different way in many parts of the body (14). The PPAR activators seem to inhibit tumor growth by several mechanisms including inhibition of angiogenesis (15).

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PPAR $\gamma$  and PPAR $\alpha$  have a wide range of events involving the vasculature, including atherosclerotic plaque formation and stability, vascular tone, and angiogenesis (16).

Recent studies showed that the PPAR- $\gamma$  pathway may be a therapeutic target for numerous diseases in which excessive angiogenesis is implicated, including cancer (17, 18).

Hence, in this study, we investigated whether the anti-angiogenic effect of PPE is partly attributable to PPARs activation in the HUVECs. For this purpose, we used T0070907 as a selective ligand for PPAR $\gamma$ , and GW6471 as a PPAR alpha specific inhibitor (19, 20).

## Materials and Methods

### Preparation of pomegranate peel ethanol extract

Fresh pomegranate fruit was purchased from Agriculture research center of Isfahan, Iran. Pomegranate peels were dried and powdered and extracted with ethanol 70% containing 1% acetic acid at room temperature for 24 hr. The extract was prepared as described previously (6).

Total anthocyanin content was determined by the pH differential method (21). High-performance thin-layer chromatography method for the determination of ellagic acid as the major component of pomegranate was done at 280 nm using a TLC Scanner 3 (CAMAG, Muttenz, Switzerland) (22).

### Cell culture

The HUVECs (National cell bank of Iran affiliated to Pasteur Institute, Tehran, Iran) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 1% antibiotics (100 units/ml penicillin and 100  $\mu$ g/ml streptomycin), and 10% fetal bovine serum. The HUVECs were grown at 37 °C in humidified air with 5% CO<sub>2</sub> incubator. When the cells were 70–80% confluent, they were treated with 0.25% trypsin and passaged to a new culture. The cells were grown at 37 °C in humidified air with 5% CO<sub>2</sub> incubator. The experiments were conducted with cells from passages 2 to 6.

### Angiogenesis assays

A total of 100  $\mu$ L Matrigel Basement Membrane Matrix (Invitrogen, USA) was coated for 30 min at 37°C within a 24-well plate. The cells were detached by trypsin- EDTA, and after neutralization of trypsin, cells were resuspended in Medium 200PRF containing 10% FBS; then, HUVECs ( $1 \times 10^5$ ) in 4 groups were seeded on the Matrigel.

Each well was treated with different treatments, which included PPE (10  $\mu$ g/ml), PPE + PPAR- $\gamma$  antagonist (T0070907; 50  $\mu$ mol/l), PPE + PPAR $\alpha$  antagonist (GW6471; 50  $\mu$ mol/l), and the control group (DMSO 0.1 %).

After 24 hr of incubation, Calcein AM cell-permeable dye was added to each well and

photographed with a fluorescent microscope. Finally, tube length, size, and junction were quantified using the AngioQuant v1.33 software (The MathWorks, Natick, MA, USA).

### Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR)

Total RNA was extracted from HUVEC using an RNeasy Mini plus Kit (Qiagen, Valencia, CA, USA), following the manufacturer's protocols.

cDNA was synthesized using RevertAid<sup>TM</sup> Reverse Transcriptase (Fermentas, Vilnius, Lithuania) with oligo-dT primers as described before (6). QRT-PCR was performed using specific primers for VEGFA and GAPDH (as an internal control) mRNAs with the Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Vilnius, Lithuania) and run on the Rotor-gene 6000 (Qiagen, Hilden, Germany). The PCR cycling conditions for the genes included an initial denaturation step at 95 °C for 10 min, followed by 45 amplification cycles consisting of denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec and an extension at 72 °C for 30 sec.

Primers for qRT-PCR:

GAPDH: AATGCATCCTGCACCACCAA

GAPDH: GTAGCCATATTCATTGTCATA

VEGF-A: GCAGAATCATCACGAAGTGG

VEGF-A: GCATGGTGATGTTGGACTCC

### Statistical analysis

The experiments were performed in duplicate and replicated three times. At last, using SPSS 15 tests in one way ANOVA and LSD as *post hoc* (Bonferroni) analysis were performed. Values of  $P < 0.05$  were considered statistically significant.

## Results

### Antioxidant assay

Using the pH differential method for freeze dried PPE, its anthocyanin content equivalent to cyaniding-3-glucoside was detected as  $1.77 \pm 1.80$ .

Dried extract of the pomegranate peel was standardized to contain  $1.57 \pm 0.128$  (g/100 g) ellagic acid.

### Angiogenesis

For the assessment of the anti-angiogenic activity of PPE, HUVECs were added on Matrigel then treatment was started. Dimethyl sulfoxide (DMSO) 0.1% was used as control. The results showed that PPE (10  $\mu$ g/ml) significantly suppressed the formation of tube-like structures (size, length, junction) compared the control ( $P < 0.05$ ) (Figure 1 and Table 1). PPAR antagonists completely antagonized the formation of the tube-like structures of HUVEC cells. There was no significant difference between the control group and PPE plus antagonists group ( $P > 0.05$ ).

**Table 1.** Endothelial tube size, length, and junction after treatment in different groups on Matrigel

	Size	Length	Junction
Control	32860±3404	8546±209	153±9
PPE (10 µg/ml)	18934±1800 #	4743±1214#	47±24#
PPE+T0070907	43074±815*	8767±695*	214±52*
PPE+GW6471	35708±3297	8592±1382	215±23*
T0070907	27460±815	8830±695	214±52
GW6471	19149±990	6650±835	82±40

# Significant decrease compared to the control group ( $P<0.05$ ); \* Significant increase compared to the PPE group ( $P<0.05$ )

### Effects of PPAR- $\gamma$ and $\alpha$ antagonists on tube formation

To examine whether PPE inhibits angiogenesis through PPARs, we used PPAR antagonists. For this purpose, we used T0070907 as PPAR- $\gamma$  antagonist and GW6471 as PPAR $\alpha$  antagonist. Interestingly results have shown that treatment of the endothelial cells with PPE plus antagonists has led to significantly increased tube formation (Figure 1, Table 1).

### PPE inhibits expression of VEGF mRNA in HUVECs but this action is suppressed by PPAR antagonists

After optimization of the qRT-PCR, expression of VEGFA and GAPDH genes was determined in the treated and control cells. Relative expression of VEGF gene was determined by dividing its expression amount to that of the GAPDH gene. PPE (10 µg/ml) significantly inhibits VEGF mRNA expression ( $P<0.05$ ). Interestingly the cells that were treated by both of PPARs antagonists suppressed the PPE activity and there was significant increase in VEGF mRNA expression compared to control and PPE groups ( $P<0.05$ ). Interestingly, these results confirm the results of Matrigel assay.

### Discussion

The present study demonstrates that when HUVECs were treated simultaneously by PPE and PPAR $\alpha$ - or PPAR $\gamma$ -antagonist, the anti-angiogenic effect of PPE suppressed and EC tube formation increased. Our qRT-PCR results also corroborate the results of Matrigel assay. After treatment by PPARs

antagonists and PPE, increased VEGF mRNA expression raises the possibility that anti-angiogenic effect of pomegranate may be suppressed through the PPAR pathway.

We have recently demonstrated that PPE can suppress the formation of tube-like structures in (200, 300, and 400 µg/ml) doses(6), therefore, we examined the PPE (10 µg/ml) to find the minimal effective dose and for assessment of PPE anti-angiogenic potential in lower doses and the second probable mechanism.

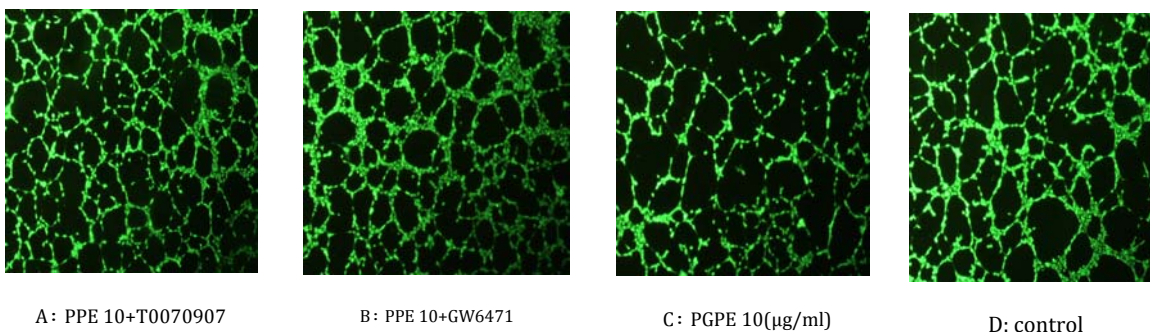
Pomegranate peel is rich in estrogenic flavonoids that have been shown to be anti-angiogenic.

Previous studies indicate that pomegranate compartments, inhibit angiogenesis in cancer and human umbilical vein endothelial cell lines via downregulation of vascular endothelial growth factor (23).

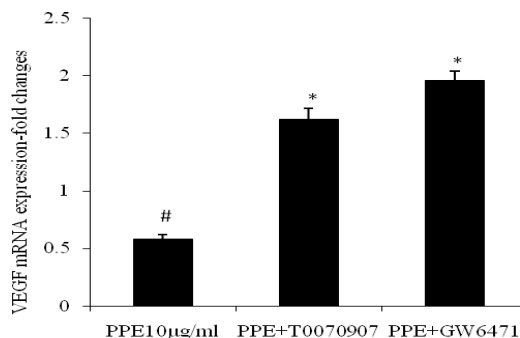
Several *in vitro* and *in vivo* studies proposed that selective activation of PPAR $\alpha$  and PPAR $\gamma$  promotes a vigorous angiogenic process by a mechanism dependent on VEGF stimulation (24, 25).

We defined the role of PPAR antagonist in suppressing the anti-angiogenic effect of PPE. The treatment of endothelial cells with PPE simultaneous PPAR antagonists showed that anti-angiogenic effects of PPE were inhibited. Further PPE anti-angiogenic properties may be involved by the PPARs-dependent mechanism.

PPAR $\alpha$  and PPAR $\gamma$ , which are both expressed in endothelial cells, have several well-characterized roles in endothelial cells, including anti-inflammatory, antiproliferative, and anti-angiogenic effects (26, 27).



**Figure 1.** Morphological features of Human Umbilical Vein Endothelial Cells after different treatments. HUVECs were plated on a well coated with 100 µl of Matrigel basement membrane matrix. After they were treated with PPE for 24 hr, the cells were dyed and photographed with a Nikon camera attached to a fluorescent microscope at  $\times 10$  magnification. A: HUVECs were treated with PPE +T0070907, B: HUVECs were treated with PPE +GW6471, C: Treatment of the cells with PPE, D: control group



**Figure 2.** Quantitative analysis of mRNA expression of vascular endothelial growth factor in Human Umbilical Vein Endothelial Cells by real-time RT-PCR. After treatment of HUVECs by PPE alone or PPE with PPAR antagonists, total RNA was extracted. The relative expression of VEGF was normalized to GAPDH levels measured in the same RNA preparation. Data shown are from three independent experiments analyzed in duplicate. # Significant decrease compared to the control group.\* Significant increase compared to the control group

PPAR $\alpha$  has been shown to regulate the expression of the VEGF. Biscetti *et al* have indicated that WY14643 (PPAR $\alpha$  agonist) promotes corneal angiogenesis *in vivo* and enhances endothelial tubulogenesis *in vitro* (28).

PPAR $\alpha$  ligands might act as potent anti-angiogenic factors, for example, endothelial cells proliferation was avoided by fenofibrate through inhibiting cyclooxygenase-2 expression (29, 30). PPAR $\alpha$  agonists were shown to inhibit endothelial VEGFR2 expression by inhibiting Sp1-dependent promoter binding and transactivation (31). Finally, we have shown that PPAR $\alpha$  and  $\gamma$  agonists inhibit endothelial tube formation (32).

Inhibition of VEGF, and VEGF receptor expression and mitogen-activated protein kinase-dependent activation are probable mechanisms of anti-angiogenic actions of PPAR $\gamma$  (33, 34). However, the anti-angiogenic mechanism of PPAR $\gamma$  activation in the endothelial cells remains currently unclear.

Hontecillas *et al* in their studies demonstrate that punicic acid, which is a conjugated linolenic acid isomer frequently found in pomegranate, binds and robustly activates PPAR $\gamma$ , increases PPAR $\gamma$ -responsive gene expression (12), furthermore, other pomegranate compartments such as flowers, have been shown to activate PPARs.

These findings indicate for the first time that PPAR $\gamma$  and  $\alpha$  might be the molecular targets for PPE extract and provide a better understanding of the potential mechanism of the anti-angiogenic action of PPE.

Further *in vivo* and *in vitro* studies are needed to shed more light on the underlying mechanisms of PPE beneficial effects in cancer prevention and treatment.

## Conclusion

It seems that the anti-angiogenic effects of PPE could be mediated in part through PPAR dependent pathway.

## Acknowledgment

This study was supported by Isfahan University of Medical sciences (grant no.290110), Isfahan, Iran.

## Conflicts interests

The authors declare that they have no competing interests

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