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# *Ruta graveolens* water extract inhibits cell-cell network formation in human umbilical endothelial cells via MEK-ERK1/2 pathway

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ABSTRACT

Angiogenesis is a process encompassing several steps such as endothelial cells proliferation, differentiation and migration to form a vascular network, involving different signal transduction pathways. Among these, ERK1/2 signaling mediates VEGF-dependent signaling pathway. Here we report that the water extract of *Ruta graveolens* (RGWE), widely known as a medicinal plant, is able to impair in a dose-dependent manner, cell network formation without affecting cell viability. Biochemical analysis showed that the major component of RGWE is rutin, unable to reproduce RGWE effect. We found that RGWE inhibits ERK1/2 phosphorylation and that this event is crucial in cell network formation since the transfection of HUVEC with a constitutively active MEK (caMEK), the ERK1/2 activator, induces a robust cell network formation as compared to untransfected and/or mock transfected cells and, more importantly, caMEK transfected cells became unresponsive to RGWE. Moreover, RGWE inhibits VEGF and nestin gene expression, necessary for vessel formation, and the caMEK transfection induces their higher expression. In conclusion, we report that RGWE is able to significantly impair vessels network formation without affecting cell viability, preventing ERK1/2 activation and, in turn, down-regulating VEGF and nestin expression. These findings point to RGWE as a potential therapeutic tool capable to interfere with pathologic angiogenesis.

## 1. Introduction

Angiogenesis is the formation of new blood vessels, a complex, multistep process in which endothelial cells proliferate, differentiate and migrate to form intact vascular network [1]. After birth, angiogenesis still contributes to organ growth but, during adulthood, most blood vessels remain quiescent and angiogenesis occurs only in the cycling ovary and in placenta during pregnancy. However, endothelial cells retain their ability of dividing rapidly and migrating in response to pathophysiological stimuli such as hypoxia and inflammation. As such, angiogenesis is reactivated during wound healing and repair [1–3]. But in many disorders, these responses become excessive and the balance between angiogenesis stimulators and inhibitors is tilted, resulting in an angiogenic switch. The best known condition in which angiogenic processes are imbalanced and angiogenesis is excessive, are malignancies, ocular inflammatory disorders, obesity, diabetes, cirrhosis, multiple sclerosis, endometriosis, AIDS, rheumatic diseases. Moreover, angiogenesis also promotes tumor progression and metastasis [1]. For these reasons, over the past decade, intensive efforts have been undertaken to develop therapeutic strategies to inhibit angiogenesis in cancer, ocular, joint or skin disorders [3]. The main target of the therapeutic strategies is represented by the vascular endothelial growth factor (VEGF) which plays a predominant role in angiogenesis. Indeed, VEGF facilitates endothelial proliferation and migration and regulates capillary permeability via binding to VEGFs receptors [3–6]. Up to date, several antiangiogenetic drugs have been developed and synthesized. They all act by targeting the VEGF signaling pathway. However, a significant number of patients remain unresponsive and these synthetic drugs have an unfavorable cost to benefit ratio [7,8]. Therefore, there is a strong need to develop new and affordable antiangiogenic drugs with minimal side effects to complement and combine with existing therapies. Natural products are characterized by high chemical diversity and biochemical specificity; therefore, they are appealing as lead compounds for drug discovery. Indeed, the active substances in many medicinal plant are represented by secondary metabolites such as flavonoids, polyphenoles, furocumarins and related molecules. Many of

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them have been tested for their potential therapeutic effects in several diseases such as cancer, inflammatory and cardiovascular diseases and some of them have been tested for their effects in ocular neovascular diseases specifically with very promising activity [9–11].

Recently we have demonstrated that the water extract of *Ruta graveolens* (RGWE) is able to induce death of glioma cells without affecting healthy neurons through the interaction with the ERK1/2 pathway and, thus, representing an interesting tool in anticancer therapy [12]. *Ruta graveolens* L. is a perennial plant, native of the Mediterranean region but cultivated throughout Europe and many Asian countries, including China, India and Japan. *R. graveolens,* commonly known as rue, is known as medicinal plant since ancient times and currently used, particularly in Asian countries, for treatment of various disorders such as aching pain, eye problems, rheumatism and dermatitis [13,14]. The plant contains more than 120 compounds of different classes of natural products such as acridone alkaloids, coumarins, essential oils, flavonoids and furoquinolines [15,16]. The components of *R. graveolens* species are of great interest in medicinal chemistry, as these compounds show a broad range of biological activities, and a number of them are already used in medicine. In particular, alcoholic extracts of *R. graveolens* have been tested for the treatment of inflammation and neo-angiogenesis associated with atherosclerosis in hypercholesterolemic rats [17]. The present study was aimed to assess the effects of RGWE on the ability of the human umbilical vein endothelial cells (HUVECs) to form in vitro cell-network and to investigate the modulation of VEGF and ERK1/2 activities as molecular target of the biological effects of RGWE.

## 2. Material and methods

### 2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) and endothelial cell culture media were purchased from Clonetics. HUVECs were maintained in endothelial cell growth medium: EBM2 medium (Clonetics) supplemented with 1 μg/ml hydrocortisone and 1 ng/ml epidermal growth factor, 10% FBS and penicillin/streptomycin at 37 °C in a humidified incubator (5% $CO_2$, 95% air). Cells were split at the ratio of 1:3 every passage. Cells from two to six passages, which did not show apparent differences in response to growth factors, were used in this study.

### 2.2. RGWE preparation

*R. graveolens* is not a protected species. Leaves were collected from plants conserved at the Experimental Section of Medicinal Plants at the Botanical Garden of Naples, Italy with the permission of the "Orto Botanico" director Prof. Paolo De Luca. Whole leaves were harvested before the flowering stages, during spring 2015. 250 g of leaves were chopped, boiled in 1 L of distilled water at 110 °C for 60 min. The extract was subsequently filtered through 0.22 μm filters (MILLEX®GP, MILLIPORE, Bedford, MA), frozen under liquid nitrogen and lyophilized (VirTis-SP Scientific). When necessary for the experiments, the water extract was diluted with water to standard concentration of 50 mg/ml.

### 2.3. 2D matrigel assay

A 2D matrigel assay was performed as indicated by the matrigel manufacturer (BD bioscience). Briefly, 96-well plates were plated with 50 μl matrigel and allowed to polymerize at 37 °C for 30 min. Cells were subsequently seeded on the matrigel at the concentration of $2 \times 10^4$ cells per well followed by addition of increasing doses of RGWE (0; 0,01; 0,1; 1 mg/ml) or rutin (12; 120; 300 μg/ml) and incubated for 24 h at 37 °C. Each conditional group contained 4–6 wells. The tube-like structures were photographed on each well using a phase-contrast microscope (Axiovert25, Zeiss) at a magnification of 10×. Tube-like structure in each field were imaged and an average from 3 to 5 random fields in each well was counted. To quantify the results, we counted the number of branch points, in which at least 3 tubes joined, using the software ImageJ.Ink.

### 2.4. Cell viability assay

Cellular viability was evaluated using trypan blue exclusion test and MTT assay. Trypan blue exclusion test was performed on cells subjected to tube assay. In particular, after image acquisition, cells were subjected to dispase detachment (2.4 U/ml at 37 °C for 1 h), harvested by centrifugation at 1500 rpm for 3 min and resuspended in 0.5 ml of PBS. 0.2 ml of cell suspension were added to 0.5 ml of PBS and 0.3 ml of 0.4% of Trypan blue solution (Lonza). After 5 min at room temperature, cells were counted in a Burker's chamber. For each of the conditions tested for matrigel assay, we performed parallel MTT assay to evaluate cell viability with the same passage and origin of the cells. In particular, cells were seeded at $3 \times 10^5$ cells/well in a 24 well plate in the presence or absence of increasing doses of RGWE (0; 0,01; 0,1 and 1 mg/ml), and cell proliferation assessed after 24 h. According to manufacturer's recommendations, 50 μl of 3-(4,5 dymethylthiazol-2-il)-2,5 dyphenyl-2H-tetrazolium bromide (MTT; Sigma Aldrich, USA) reagent (5 mg/ml in PBS) was added to each well and, then, the cells were incubated at 37 °C for three hours. One volume (500 μl) of Stop mix solution (20% SDS in 50% dimethyl formamide) was added to each well and incubated at room temperature for a minimum of 1 h. The plate was read at 550 nm and at 630 nm as the reference wavelength. Same volume of medium without cells was used as blank. Results are expressed as OD.

### 2.5. Biochemical characterization of RGWE

#### 2.5.1. RP-HPLC analysis

150 μg of the liophylized RGWE was subjected to RP-HPLC analysis by using a Breeze HPLC System (Waters, Milford, MA, USA) equipped with a UV detector. The sample was loaded onto a Symmetry 300 C18, column (5 μm, 300 Å, 0.46 × 15 cm, Waters). Elution was obtained using a linear gradient of 0.1% TFA (solvent A) and acetonitrile containing 0.1% TFA (solvent B) from 5% to 65% of solvent B over 60 min. The detection wavelengths were set at 280 and 360 nm. Standard rutin (Sigma Aldrich, USA) dissolved in solvent A (1 mg/ml) was also injected as reference compound.

#### 2.5.2. LC ESI-Q/TOF mass spectrometry analysis

RGWE (0.25 g/ml) was diluted 1:10 in 0.1% formic acid and analysed by liquid chromatography (LC) mass spectrometry (MS) using a quadrupole time of flight (ESI-Q/TOF) mass spectrometer (Q-TOF Micro, Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source. Electrospray mass spectra and tandem MS/MS data were acquired in the positive ion mode with a source temperature of 80 °C and nitrogen used as drying gas (flow rate about 50 L/h). The capillary source voltage and the cone voltage were set at 3500 and 43 V, respectively. Analytes were separated by means of a modular CapLC system (Waters, Manchester, UK), directly connected with the ESI source. The sample was loaded onto a C-18 precolumn (5 mm length x 300 μm ID) at a flow rate of 20 μl/min and desalted for 5 min with a solution of 0.1% formic acid. Analytes were then directed onto a symmetry-C18 analytical column (10 cm × 300 μm ID) using 5% $CH_3CN$, containing 0.1% formic acid at a flow rate of 5 μl/min. Elution was carried out increasing the $CH_3CN$/0.1% formic acid concentration from 5% to 55% over 60 min. Electrospray mass spectra and tandem MS/MS data were acquired on the Q-TOF mass spectrometer operating in the positive ion mode. MS/MS data were acquired in the data directed analysis (DDA) MS/MS mode. MS/MS fragmentation spectra were collected from $m/z$ 50 to $m/z$ 1600. The MS/MS data were processed by MassLynx 4.0 software (Waters, Manchester, UK). Compound identification was performed by using the MassBank high quality mass