Phenolic and anthocyanin fractions from wild blueberries (*V. angustifolium*), differentially modulate endothelial cell migration through RHOA and RAC1.

Wild blueberries modulate cell migration.

Panagiotis Tsakiroglou[1], James Weber[1], Sharon Ashworth[2], Cristian Del Bo'[3] and Dorothy Klimis-Zacas[1,*]

[1] School of Food and Agriculture, University of Maine, Orono, Maine, 04469, USA; panagiotis.tsakiroglou@maine.edu (P.T.); jaweber@maine.edu (J.W.); dorothea@maine.edu (D.K.Z.)

[2] School of Biology and Ecology, University of Maine, Orono, Maine, 04469, USA; sharon.ashworth@maine.edu (S.A.)

[3] Department of Food, Environmental and Nutritional Sciences, Division of Human Nutrition, Università degli Studi di Milano, 20133 Milan, Italy; cristian.delbo@unimi.it (C.D.B.)

***** Correspondence: dorothea@maine.edu; Tel.: +1-207-581-3124

Acknowledgement: This study was funded by the Wild Blueberry Association of North America. We would like to thank FutureCeuticals (Momence, IL, USA) for providing the wild blueberries. Maine Agricultural and Forest Experiment Station Publication Number. Some equipment used in this study was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health P20GM0103423.

Conflict of interest: The authors declare no conflict of interest.

Abstract: The present study investigates the effect of anthocyanin (ACN), phenolic acid (PA) fractions and their combination (ACNs:PAs) from wild blueberry powder *(Vaccinum angustifolium)* on the speed of endothelial cell migration, gene expression and protein levels of RAC1 and RHOA associated with acute exposure to different concentrations of ACNs and PAs. Time-lapse videos were analyzed and endothelial cell speed was calculated. Treatment with ACNs at 60 μg/mL inhibited endothelial cell migration rate ($p \leq 0.05$) while treatment with PAs at 0.002 μg/mL ($p \leq 0.0001$), 60 μg/mL ($p \leq 0.0001$) and 120 μg/mL ($p \leq 0.01$) significantly increased endothelial cell migration rate compared to control. Moreover, exposure of HUVECs to ACNs:PAs at 8 μg/mL:8 μg/mL ($p \leq 0.05$) and 60 μg/mL:60 μg/mL increased ($p \leq 0.001$) endothelial cell migration. Gene expression of RAC1 and RHOA significantly increased 2 h after exposure with all treatments. No effect of the above fractions was observed on the protein levels of RAC1 and RHOA. Findings suggest that endothelial cell migration is differentially modulated based on the type of blueberry extract (ACN or PA fraction) and is concentration-dependent. Future studies should determine the mechanism of the differential action of the above fractions on endothelial cell migration.

Keywords: Human umbilical vein endothelial cells (HUVECs); cell migration; RAC1; RHOA; anthocyanins; phenolic acids; wild blueberries (*Vaccinium angustifolium)*

Introduction

One fundamental process common to cell morphogenesis, physiology and development, immune function, and disease is cell migration (Szabo & Mayor, 2015; Zegers & Friedl, 2014). The wound healing process involves hemostasis, inflammation, cell differentiation, proliferation and cell migration which promotes angiogenesis and finally

tissue remodeling (Guo & Dipietro, 2010). Since wound healing is a cellular response to injury, many cell types, including endothelial cells, are stimulated and coordinated in this complex biological phenomenon to perform a balanced wound healing process (Guo & Dipietro, 2010; Kolluru, Bir, & Kevil, 2012). Several factors can affect wound healing and can be classified as local or systemic (Guo & Dipietro, 2010). Some of the systemic factors are: age, gender, stress, alcohol, smoking, malnutrition, obesity and diseases (Guo & Dipietro, 2010). Chronic diseases such as diabetes lead to impaired wound healing, a result of unbalanced angiogenesis (Abaci et al., 1999; Kolluru et al., 2012; Waltenberger, Lange, & Kranz, 2000). Reduced blood flow to the extremities, decrease in endothelial cell proliferation and angiogenesis result in improper response of diabetic patients to injury (Kolluru et al., 2012).

The lowbush blueberry (*Vaccinium angustifolium)* has been ranked as one of the richest food sources of bioactive compounds (polyphenols) such as anthocyanins (ACNs) and phenolic acids (PAs) generally found in fruits and vegetables (Del Bo, Cao, et al., 2016). The antioxidant activity of wild blueberries is a result of ACNs, procyanidins, chlorogenic acid, and other phenolic compounds (Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). ACNs from wild blueberries are primarily composed of delphinidin, malvidin, petunidin, cyanidin and peonidin (Bushway, McGann, Cook, & Bushway, 1983; Del Bo et al., 2010). Their phenolic content is often influenced by growing practices, location and harvesting methods (Del Bo, Cao, et al., 2016; Taverniti et al., 2014).

Numerous *in vivo* and *in vitro* studies have documented the beneficial effects of wild blueberry consumption on inflammation and cardiovascular disease (CVD) as well as many other chronic diseases (Del Bo, Cao, et al., 2016; Del Bo et al., 2010; Del Bo, Roursgaard, et al., 2016; Kay & Holub, 2002; Kristo, Kalea, Schuschke, & Klimis-Zacas, 2010; Riso et al., 2013; Skrovankova et al., 2015; Vendrame, Kristo, Schuschke, & Klimis-Zacas, 2014). Only a few studies have documented the effect(s) of single ACNs, PAs (nasunin, delphinidin, pelargonidin, ellagic acid and epigallocatechin-3 gallate) and fractions from different berries on cell migration, angiogenesis and wound healing (Lamy, Akla, Ouanouki, Lord-

Dufour, & Beliveau, 2012; Lamy et al., 2006; Matsunaga, Chikaraishi, Shimazawa, Yokota, & Hara, 2010; Son, Jeong, et al., 2014).

Single ACNs such as nasunin ((Delph inidin-3-(p-coumaroylrutinoside)-5-glucoside) was found to inhibit HUVEC proliferation rate at 200 µM, 100 µM and 50 µM (16) and Delphinidin (Dp) to increase vascular endothelial growth factor (VEGF)-induced tube formation of HUVECs (Lamy et al., 2006). Diet-delivered polyphenols (apigenin, delphinidin, ellagic acid and epigallocatechin-3 gallate) inhibited endothelial cell migration, proliferation and tubulogenesis through the JAK/STAT3 and MAPK signaling pathways (Lamy et al., 2012). The inhibitory effect of ACNs (pelargonidin and its glucoside-conjugated form, pelargonidin-3-glucoside (P3G)), on cell proliferation and smooth muscle cell migration was documented, with FAK being a significant molecular target of ACNs (Son, Jeong, et al., 2014). ACNs extracted from black soybean seeds coats were found to stimulate wound healing in chronic wounds by reducing the inflammatory state of the wound (Nizamutdinova et al., 2009)

Evidence documents the importance of small G proteins in cell motility (Brown, Del Re, & Sussman, 2006; Heng & Koh, 2010; Jaffe & Hall, 2002; Vial, Sahai, & Marshall, 2003). RHOA, RAC and CDC42 are known members of the RHO family and they play a key role in the actin cytoskeleton (Vial et al., 2003). During cell migration RAC is involved in the formation of lamellipodia at the leading edge of the tip/migrating cells and RHOA is a regulator of actin stress fibers and is required during focal adhesions (Vial et al., 2003). *In vitro* (human microvascular endothelial cells and HUVECs) and *in vivo* (C57BL/6 mice) studies have documented the role of berry extracts and PAs (gallic acid from red raspberries) on cell migration and angiogenesis promoting or inhibiting expression of RHO GTPases, receptors (VEGFR2/NRP1) and other molecules (AKT, ERK 1/2, VEGFA and p38) involved in molecular pathways controlling those functions (Matsunaga et al., 2010; Sousa et al., 2016; Tanaka et al., 2012).

Since there is a paucity of research on the role of ACNs and PAs on endothelial cell migration in relation to wound healing, the **goal** of this study is to investigate the effect of

ACN and PA fractions and their combination (ACNs:PAs) from wild blueberry powder *(Vaccinum angustifolium)* to determine whether they operate to alter cell migration.

The **objectives** of this study are to determine the effect of ACNs, PAs and their combinations: **a.** on proliferation rate of the endothelial cells **b.** speed of endothelial cell migration after acute exposure to the above compounds and **c.** changes in gene expression and protein levels of RAC1 and RHOA proteins, critical for cell migration.

Materials and Methods

Cell Culture

Human umbilical vein endothelial cells (HUV-EC-C [HUVEC] (ATCC® CRL-1730™)) were purchased from the American Type Culture Collection (ATCC®) Manassas, VA, USA. Human umbilical vein endothelial cells were maintained in F-12K medium (Kaighn's modification of Ham's F-12 medium) (ATCC® 30-2004™) with 10 % fetal bovine serum (FBS) (ATCC® 30-2020™) and 1 % penicillin-streptomycin solution (ATCC® 30-2300™), heparin 0.1 mg/mL (Sigma, H3149) and endothelial cell growth supplement (ECGS) 0.03 mg/mL (Sigma E2759). The culture vessel for the growth of the cells was the Corning® T-75 flask (catalog #3276). The culture conditions for the cell line were air 95 %, carbon dioxide (CO_2) 5 %, 90 % of relative humidity (RH) and a temperature of 37 ^{o}C.

Extraction and analysis of ACNs and PA fractions from wild blueberry powder

Wild blueberries (WB) were provided as a composite by Wyman's (Cherryfield, Maine, USA) and processed following standard procedures to obtain a freeze-dried powder (FutureCeuticals, Momence, Ill., USA) (Vendrame et al., 2014). Vacuum-packed plastic bags with the wild blueberry powder were stored at −20 °C until use. The wild blueberry powder had a total content of 1.5% w/w of anthocyanins, with malvidin-3-galactoside and peonidin-3-glucoside being the most abundant forms, as previously reported (Fracassetti et al., 2013). From the freeze-dried wild blueberry powder three fractions were isolated:

1. Phenolic-rich fraction (ethyl acetate soluble, containing mainly chlorogenic acid) **2.** Anthocyanin-rich fraction (methanol soluble fraction, containing mainly anthocyanins) and **3.** Water soluble fraction.

The extraction of ACNs and PAs from the WB powder was performed according to the method previously described (Del Bo, Cao, et al., 2016; Taverniti et al., 2014). Determination of total phenolic concentration was conducted by the Folin-Ciocalteu method (Bower, Real Hernandez, Berhow, & de Mejia, 2014; Velioglu, Mazza, Gao, & Oomah, 1998; Wan et al., 2011). For the determination of the ACN fraction concentration, a pH differential method was used (Lee, Durst, & Wrolstad, 2005).

The concentration of ACN and PA in the wild blueberry fraction was determined by HPLC. The system consisted of an Alliance mod. 2695 (Water, Milford, MA) equipped with a mod. 2998 photodiode array detector (Waters) as previously reported (Del Bo, Cao, et al., 2016; Taverniti et al., 2014).

Cell proliferation and cytotoxicity assay

The cell growth curve experiment provided information on the doubling time of the HUVECs. The ACNs and PAs cytotoxicity assay was conducted so that the optimum concentration and exposure time of the active compounds was used for the rest of the experiments. Different concentrations (0.001 μg/ml - 1000 μg/ml for ACNs and 0.001 μg/ml – 500 μg/ml for the PAs) and different exposure times were tested (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h). Cell proliferation and cytotoxicity assays were conducted by using the alamarBlue assay (Life Technologies, DAL1025). Cells for both cell proliferation and cytotoxicity assays were measured by using Synergy 2 multiwell plate reader (Bio-Tek Instruments Inc., Winooski, VT) with excitation⁄emission (530 nm-560 nm ⁄ 590 nm) as previously described (Stoddart, 2011).

Cell migration

The effect of ACNs, PAs and their combination on cell migration was evaluated by the IBIDI Culture–Insert (Ibidi, Munich, Germany). The culture inserts were seeded with $5x10^5$ cells/mL with a final volume in each insert chamber of 70 μl. The IBIDI Culture–Insert was

placed in the incubator for 24 hours. After cells reached ≥90% confluence they were treated with the PA and ACN fractions and their combination at concentrations as determined by the results of the cytotoxicity experiment (0.002 µg/mL, 8 µg/mL, 15 µg/mL, 60 µg/mL and 120 µg/mL). Cells in the treated (ACN and PA fractions and combination, n=7 replicates) and untreated (control, n=10 replicates) wells were observed under an inverted phase-contrast optical microscope (Nikon TS100) until endothelial cells fully migrated into the free area. At the end of each experiment the speed of closure was calculated as the cell migration rate ($v_{migration}$ in µm/hr). Analysis was conducted with the TScratch software as described in detail by Geback and Jonkman (Geback, Schulz, Koumoutsakos, & Detmar, 2009; Jonkman et al., 2014).

Gene expression, real-time RT-PCR analysis

Endothelial cells were cultured and maintained as described previously. HUVECs were treated with ACN and PA fractions and their combination for 2 h and 6 h. mRNA was isolated using the RNeasy Kit (Qiagen) and DNase Digestion (Qiagen) was used for RNA purification. QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis and removal of genomic DNA. A two-step RT-PCR was followed using a CFX96 (BioRad) PCR system. A 20 µl PCR reaction volume was performed using TaqMan gene expression master mix (Invitrogen) and TaqMan probes (Invitrogen): RHOA (Hs00357608_m1), RAC1 (Hs01902432_s1) and GAPDH (Hs99999905_m1).

Active GTPase pull-down assay

For immunoprecipitation, cells were lysed using the Cell Signaling active RHO detection kit (8820) and active RAC1 detection kit (8815) following manufacturer's instructions and treated with GTPγS (positive control) and GDP (negative control). Moreover, in the 1X Lysis/Binding/Wash Buffer, phenylmethylsulfonyl fluoride (PMSF) (Cell Signaling, 8553) was used at a concentration of 1 mM. Concentration of total protein was measured using BCA assay (Thermo Fisher, 23225). Immunoprecipitated materials and total protein samples were resolved by 4 - 20 % mini-protean TGX stain-free protein

gel (BioRab, 4568094). After transfer using Trans-Blot Turbo System (BioRad, 1704150), LF-PVDF membranes (BioRad, 1704274) were blotted with anti-RAC1 (1:1000, Cell Signaling, 8631) and anti-RHOA (1:667, Cell Signaling, 8789). Proteins were detected with antibodies specific for either mouse or rabbit IRDye® 800CW Goat anti-Mouse IgG, (1:15000, Li-COR, 925-32310) and IRDye® 800CW Goat anti-Rabbit IgG, (1:15000, Li-COR, 925-32211) using the Li-COR Odyssey imaging system (Li-COR Biosciences). All assays were repeated at least four times in independent experiments.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA). For the cell migration experiments, the data were analyzed by one-way ANOVA. For post-hoc comparisons, Fisher's least significant difference (LSD) test was used. For the gene expression and western blot experiments a two-tail Mann-Whitney U-test was performed to compare the control to ACNs (60 μg/mL), while one-way ANOVA was used for PAs (0.002 μg/mL, 60 μg/mL and 120 μg/mL) and combination (ACNs:PAs) (8 μg/mL: 8 μg/mL and 60 μg/mL: 60 μg/mL) groups. For post-hoc comparisons, Fisher's least significant difference (LSD) test was used. All data in the graphs are expressed as mean ± SEM. A p-value of <0.05 was considered significant. For the cell migration experiments, the control included 10 replicates (n=10) and each treatment group (ACNs, PAs and ACNs:PAs) included 7 replicates (n=7), while for gene expression experiments the control included 10 replicates (n=10) and each treatment group (ACNs, PAs and ACNs:PAs) included 10 replicates (n=10). Lastly for IP experiments the control included 4 replicates (n=4) and each treatment group (ACNs, PAs and ACNs:PAs) included 4 replicates each (n=4).

Results

Characterization of ACN and PA fractions

HPLC profile analysis of the ACN fraction is reported in Figure 1. The total ACN concentration was 45.11 ± 0.35 mg/mL with 15 different ACNs detected. Malvidin glucosides were higher in concentration (26.5 %) followed by malvidin galactoside (14.8 %) while delfinidin glucoside (8.9 %), petunidin glucoside (8.2 %) and cyanidin glucoside (7.4 %) followed. The PA fraction contained mainly chlorogenic acid (10.23 ± 1.8 mg/mL) with traces of ferulic and caffeic acids (Figure 1).

Cell proliferation assay

The HUVEC proliferation rate was calculated to seed the appropriate number of cells at the 96-well plate for the cell cytotoxicity assay. Quadruplicate samples were used for each different concentration of ACNs and PAs.

No significant differences were observed in HUVEC proliferation rates during the different exposure times tested (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h and 72 h) at the concentrations tested, (0.001 μg/ml - 1000 μg/ml for ACNs and 0.001 μg/ml – 500 μg/ml for the PAs) compared to the control. The highest concentration of ACNs (1000 μg/mL) only, documented inhibition of the proliferation rate of endothelial cells (data not shown).

Cell migration assay

After determining the appropriate concentrations of ACN and PA fractions that were not cytotoxic to the HUVECs, the cell migration assay was performed to evaluate the effect of five concentrations (0.002 μg/mL, 8 μg/mL, 15 μg/mL, 60 μg/mL and 120 μg/mL) of ACNs, PAs and their combination (ACNs:PAs). During treatment with ACNs (Figure 2A) endothelial cell migration speed was reduced at the 60 μg/mL compared to control ($p \leq 0.05$). No statistically significant differences in cell migration were detected at ACN concentrations of 0.002 μg/mL, 8 μg/mL, 15 μg/mL and 60 μg/mL. Treatment with PAs (Figure 2B) promoted endothelial cell migration. Exposure of the endothelial cells to 0.002 μg/mL, 60 μg/mL ($p \leq 0.0001$) and 120 μg/mL ($p \leq 0.01$) PAs, significantly increased the speed of endothelial cell migration compared to control. Additionally, combination of both ACNs and PAs (ACNs:PAs) at 8 μg/mL:8 μg/mL and 60 μg/mL:60 μg/mL respectively,

significantly increased endothelial cell migration rate compared to control (Figure 2C). Statistical differences among treatment groups were not detected at any of the other tested ACN:PA combinations.

Visual depictions of cell migration from all treatments of ACNs, (60 μg/mL), PAs (60 μg/mL) and combination of both fractions (60 μg/mL:60 μg/mL) (Figure 3) can be observed by frames from exposure of HUVECs at different time points (0 h, 2 h, 4 h, 6 h and 8 h). In this montage, pictures have been taken from the time-lapse video at the above-mentioned time-points and the cell migration border is marked with white dotted lines so that the progression of endothelial cell migration towards the empty area can be clearly observed.

Gene expression and immunoprecipitation (IP) assays

After determining the concentrations that documented a significant effect of endothelial cell migration, gene expression and IP experiments were important to reveal whether there was a change in RAC1 and RHOA genes and protein levels. Concentrations tested were ACNs at 60 μg/ml, PAs at 0.002 μg/ml, 60 μg/ml and 120 μg/ml and ACNs:PAs at 8 μg/ml and 60 μg/ml. Gene expression for RAC1 (Figure 4A) and RHOA (Figure 4B) was tested at two hours post treatment (Figure 4). After 2 hours of HUVEC exposure to ACNs, gene expression of RAC1 (Figure 4A) increased almost fourfold compared to control. Similarly, exposure to PAs for 2 hours significantly upregulated RAC1 expression compared to control, at all concentrations tested. Gene expression for RHOA (Figure 4B) also shows a similar pattern as RAC1.

Quantitative western blot analysis of total RAC1 (Figure 5A) documented inhibition of total RAC1 when cells were treated with PAs at 0.002 μg/ml, while total RAC1 increased at ACNs:PAs at 8 μg/ml after 2 h of exposure only.

Quantitative western blot analysis of RHOA (Figure 5B) documented increased levels of RHOA when cells were treated with PAs at 0.002 μg/mL (**: $p \leq 0.01$) and ACNs:PAs at 8 μg/mL (*: $p \leq 0.05$) 2 hours post treatment.

Discussion

The present study investigated the effects of different concentrations of extracts from wild blueberries on endothelial cell migration speed, gene expression and protein synthesis of RAC1 and RHOA as well as their effect on the proliferation rate of endothelial cells. This is a novel study that documented that ACN and PA fractions from wild blueberries differentially modulate endothelial cell migration and gene expression.

Endothelial cell migration is critical for normal wound healing processes, tissue formation and angiogenesis (Michaelis, 2014). Collective cell migration is a type of migration that plays a key role in wound healing (Mayor & Etienne-Manneville, 2016). *In vitro* experiments with HUVECs have recently unraveled possible mechanisms of collective cell migration and the role of cadherin as well as the role of RAC1, RHOA, CDC42 and F-actin in the above process (Das & Spatz, 2016).

We observed a reduction in cell proliferation 12 hours after treatment using ACNs at 1000 µg/mL when compared to controls studies documented previously (Chen et al., 2006; Kuntz et al., 2015; Lamy et al., 2012; Skrovankova et al., 2015; Speciale et al., 2013). Under our experimental conditions, we documented inhibition of cell migration when HUVECs were exposed to the ACN wild blueberry extract at 60 µg/mL and increased expression of RAC1 and RHOA at 2 hours after exposure (Matsunaga et al., 2010). In contrast exposure of HUVECs to 0.002 µg/mL, 60 µg/mL and at 120 µg/mL of the PA fraction, significantly increased speed of migration and gene expression of RAC1 and RHOA at 2 h. We also chose to test the combination of both fractions simultaneously since they are more relevant to whole berry consumption and have not been tested to date. The combination of fractions of ACNs:PAs at 8 µg/mL:8 µg/mL and 60 µg/mL:60 µg/mL significantly promoted endothelial cell migration similar to the PA fraction. The major component of the PA fraction was chlorogenic acid which is the most abundant polyphenol in the human diet (61).

Past research has shown that extracts from bilberries (25% ACNs) inhibit HUVEC cell proliferation and migration by directly inhibiting ERK 1/2 and AKT but not PLCγ pathways

(Matsunaga et al., 2010). The use of petunidin, delphinidin, cyanidin, malvidin, and peonidin inhibited smooth muscle cell migration at 20 μM by targeting focal adhesion kinase (FAK) (Son, Lee, et al., 2014), while pelargonidin at 40 μM exhibited an anti-proliferative and anti-migratory effect on smooth muscle cells (Son, Jeong, et al., 2014). Even though there are adequate number of studies on the role of ACNs on cell migration, there is paucity of research on the role of PAs and mainly focus on cancer regulation. In these studies, the PA compounds tested such as gallic, protocatechuic, syringic and chlorogenic acids inhibited the migration and angiogenesis processes by acting as potential cancer chemotherapeutic agents (Lo et al., 2011; Son, Jeong, et al., 2014). Other studies using gallic acid at ranges of 2-3.5 mM, over a 24 h period, revealed inhibition of cell motility at 6 h, 12 h and 24 h, inhibition of NF-κB activity as well as downregulation of PI3K/AKT pathway (Ho et al., 2010). In contrast, our PA fraction containing primarily chlorogenic acid, promoted endothelial cell migration and gene expression of RHOA and RAC1, molecules critical in the above process.

The differences in endothelial cell migration speed observed in our study when cells were treated with the same concentration, but different fractions, may be explained based on chemical and structural differences among them, stability of the compounds and availability to HUVEC cell receptors (Nile & Park, 2014). Additionally, the level of oxidation, glycosylation, ability to form polymeric molecules and the existence of stereoisomers may explain the results documented in our experiments (Nile & Park, 2014).

RHOA and RAC1 are critical players during cell migration; RHOA acting in the back of the cell while RAC1 in the front with CDC42. RHOA plays a key role in actin cytoskeleton formation and is involved in mechano-transduction through RHOA/RHO-kinase signaling (44, 45). RAC1 also plays a significant role in endothelial function by controlling eNOS and the phosphorylation of proteins critical for cell to cell junction such as occludin, VE-cadherin, and b-catenin (Sawada, Li, & Liao, 2010). In this study, gene expression of RAC1 and RHOA increased compared to the control after 2h exposure with all treatments while western blot analysis of RAC1 was significantly reduced after exposure to 60 μg /mL of PAs and was significantly induced after exposure to the 8 μg /mL combination. Finally,

western blot analysis of total RHOA was significantly increased after 2 h exposure to 0.002 µg/mL PAs and 8 µg/mL combination. Even though the ACNs fraction significantly inhibited HUVEC cell migration speed, gene expression of RHOA and RAC1 was significantly increased. It has been documented that RHO GTPases are responsible for cytoskeleton changes. However, since the RHO family is composed of many members there is a possibility that other members such as CDC42 may be responsible for the observations made. Since the present study evaluated collective cell migration, cadherin finger formation has been proposed to also be important for cell to cell interaction between the leading and following cell and RHOA is more critical for the formation of cadherin fingers rather RAC1, that is self-governing (Das & Spatz, 2016; Zegers & Friedl, 2014). It has also been documented that a critical activator of RAC1 is RHOG (Katoh, Hiramoto, & Negishi, 2006). In addition to that, shear stress can also act as a RAC1 activator (Tzima et al., 2002).

These are novel findings that to our knowledge have not been previously reported. Even though some studies have shown that caffeic acid at 0 – 100 µmL on smooth muscle cells significantly decreased RAC1 protein synthesis after 24 hour exposure at 10 µmol/L and 100 µmol/L compared to untreated cells (Xu et al., 2005) and protocatechuic acid downregulated the Ras/Akt/NF-κB pathway by targeting RHOB (Lin, Chen, Chou, & Wang, 2011), our PA fraction primarily contained chlorogenic acid.

The present study examined for the first time the effects of a range of low and high concentration of ACNs and PAs, obtained from wild blueberry, on cell proliferation, endothelial cell migration and gene expression of RAC1 and RHOA. Some of the concentrations used (0.002 µg/mL and 8 µg/mL) were close to the physiologically reported single compound concentrations that have been observed in the blood stream (Del Rio et al., 2013). For example, ACNs can be found in the blood stream at the concentration of 274 nM after consumption of foods in rich in these bioactives (Mullen, Edwards, Serafini, & Crozier, 2008). Research evaluating the fate of chlorogenic acids after coffee ingestion in healthy humans documented a concentration of 385 µmol isolated from ileal fluid (Stalmach et al., 2009; Stalmach, Steiling, Williamson, & Crozier, 2010). The presence of

four ACNs, delphinidin 3-O-β-rutinoside (D3R), cyanidin 3-O-β-rutinoside (C3R), delphinidin 3-O-β-glucoside (D3G), and cyanidin 3-O-β-glucoside (C3G), in the plasma of healthy humans after consumption of black currant was detected at concentrations of 73.7±35.0 nmol/L, 46.3±22.5 nmol/L, 22.7±12.4 nmol/L and 5.0±3.7 nmol/L respectively (Matsumoto et al., 2006). A range of ACNs and PAs from 0.05 to 10 μg/mL was used for lipid accumulation in macrophages (Del Bo, Cao, et al., 2016). In our experiments, the concentrations of the extracts ranged from 9 nmol/L to 579 nmol/L for ACNs and 5.6 nmol/L to 338.6 nmol/L for PAs, comparable to the above studies.

Thus, we document for the first time that different fractions extracted from wild blueberries have a significant and differential effect on endothelial cell migration which plays a key role on many physiological phenomena such as angiogenesis and wound healing. These differential effects seem to be dose and compound dependent and be orchestrated by the modulation of RAC1 and RHOA, two proteins involved in cell motility. These are novel findings that to our knowledge has not been previously reported.

Future experiments should target additional molecules associated with cell migration, shear stress and angiogenesis to reveal the mechanisms by which the above fractions confer their differential action. Additionally, evaluation of single ACN, PA compounds and their metabolites on endothelial cell migration and angiogenesis, will provide useful information on their *in vivo* role on the vascular system.

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Figure Legends

Figure. 1 HPLC profile analysis of the ACN (panel A) and PA (panel B) fractions extracted from wild blueberry (*V. angustifolium*) powder

Figure. 2 A: Endothelial cells were treated with ACNs. Treatment with ACNs at 60 µg/mL inhibited endothelial cell migration rate compared to the control (*: p≤0.05). **B:** Endothelial cells were treated with PAs. Treatment with PAs at 0.002 µg/mL, 60 µg/mL (****: p≤0.0001) and 120 µg/mL (**: p≤0.01) increased endothelial cell migration rate compared to control. **C:** Endothelial cells were treated with combination of both ACNs and PAs. Treatment with ACNs:PAs 8 µg/mL:8 µg/mL (*: p≤0.05) and ACNs:PAs 60 µg/mL:60 µg/mL (***: p≤0.001) increased endothelial migration rate compared to control.

Figure. 3 Summary of all treatments (ACNs 60 µg/mL, PAs 60 µg/mL and ACNs:PAs 60 µg/mL:60 µg/mL) that altered endothelial cell migration rate. Captured frames from time-lapse video from time-points at 0 h, 2 h, 4 h, 6 h and 8 h.

Figure. 4 A: Gene expression for RAC1 increased compared to the control for all tested concentrations two hours after treatment, ACNs 60 µg/mL (**: p≤0.01), PAs at 0.002 µg/mL (****: p≤0.0001), 60 µg/mL (****: p≤0.0001) and 120 µg/mL (****: p≤0.0001) and ACNs:PAs 8 µg/mL and 60 µg/mL (****: p≤0.0001) **B:** Gene expression for RHOA increased for all tested concentrations two hours after treatment, ACNs 60 µg/mL (***: p≤0.001), PAs at 0.002 µg/mL (***: p≤0.001), 60 µg/mL (***: p≤0.001) and 120 µg/mL (**: p≤0.01) and ACNs:PAs 8 µg/mL and 60 µg/mL (***: p≤0.001) compared to control.

Figure. 5 A: Fold change western blot analysis of total RAC1 after acute treatment (two hours) of ACNs, PAs and ACNs:PAs. Inhibition of RAC1 levels was documented with PAs at 0.002 µg/mL (*: p≤0.05) and Increased levels of RAC1 were documented with ACNs:PAs at 8 µg/mL (*: p≤0.05) after 2 h of treatment. **B:** Fold change western blot analysis of RHOA after acute treatment (two hours) of ACNs, PAs and ACNs:PAs. Increased levels of RHOA with PAs at 0.002 µg/mL (**: p≤0.01) and, PAs at 60 µg/mL (*: p≤0.05), ACNs:PAs at 8 µg/mL (*: p≤0.05) after 2 h after 2 h of exposure.

Figure 1

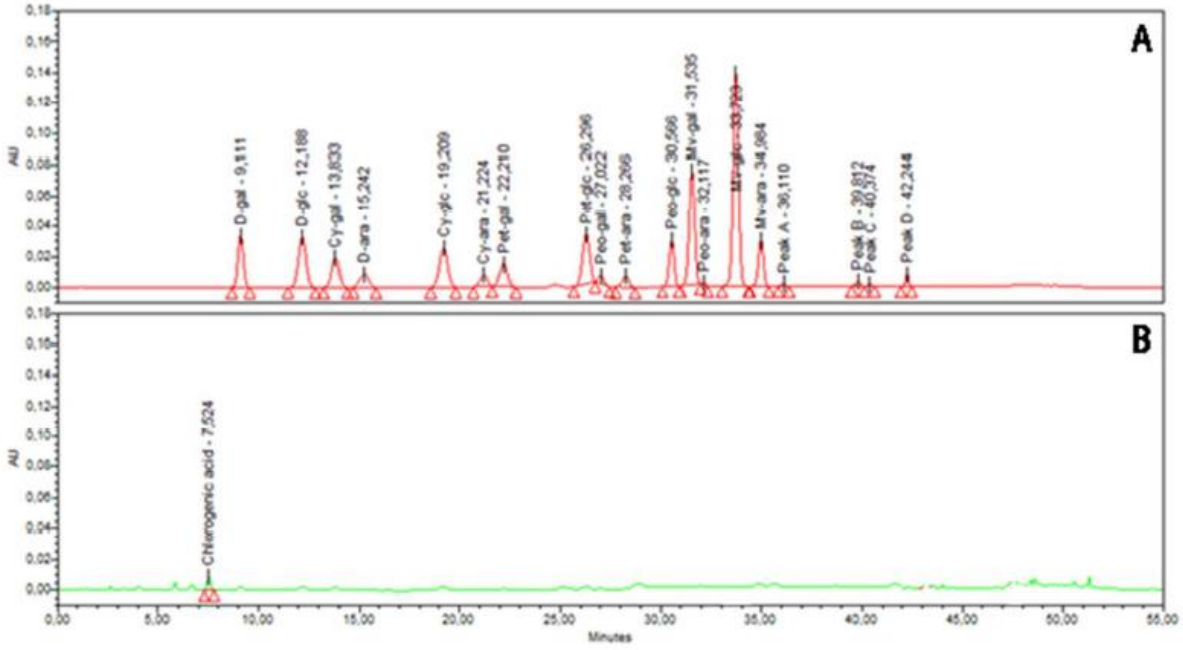

A
B
AU
D-gal - 9,111
D-glc - 12,188
Cy-gal - 13,833
D-ara - 15,242
Cy-glc - 19,209
Cy-ara - 21,224
Pet-gal - 22,210
Pet-glc - 26,296
Peo-gal - 27,022
Pet-ara - 28,266
Peo-glc - 30,566
Mv-gal - 31,535
Peo-ara - 32,117
Mv-glc - 33,393
Mv-ara - 34,984
Peak A - 36,110
Peak B - 39,812
Peak C - 40,374
Peak D - 42,244
Chlorogenic acid - 7,524
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
Minutes

Figure 2

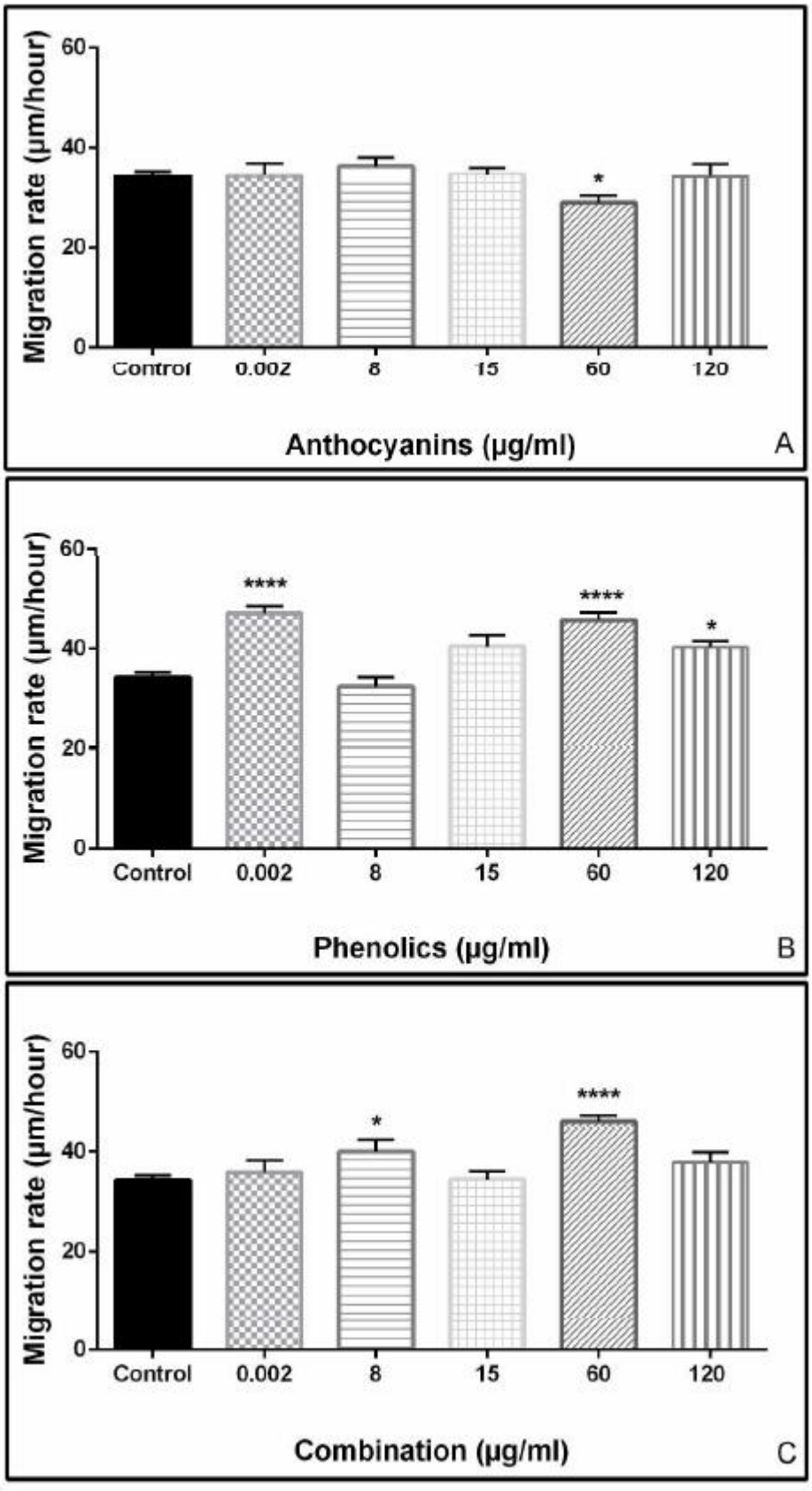

Migration rate (µm/hour)
60
40
20
0
Control
0.002
8
15
60
120
*
Anthocyanins (µg/ml)
A
Migration rate (µm/hour)
60
40
20
0
****
****
*
Control
0.002
8
15
60
120
Phenolics (µg/ml)
B
Migration rate (µm/hour)
60
40
20
0
*
****
Control
0.002
8
15
60
120
Combination (µg/ml)
C

Figure 3

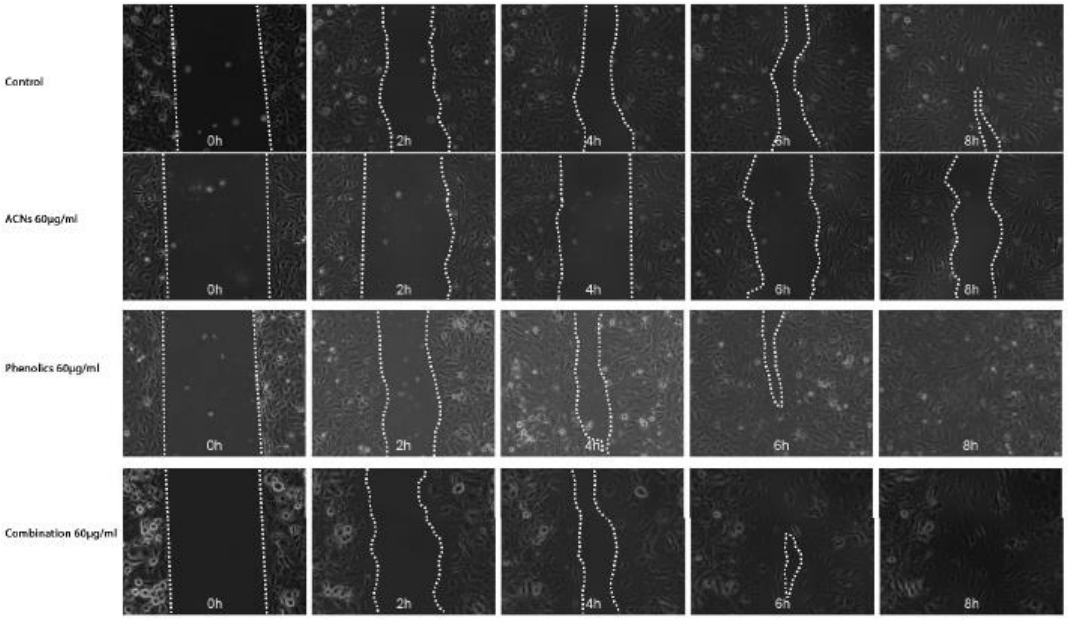

Control
ACNs 60μg/ml
Phenolics 60μg/ml
Combination 60μg/ml
0h
2h
4h
6h
8h

Figure 4

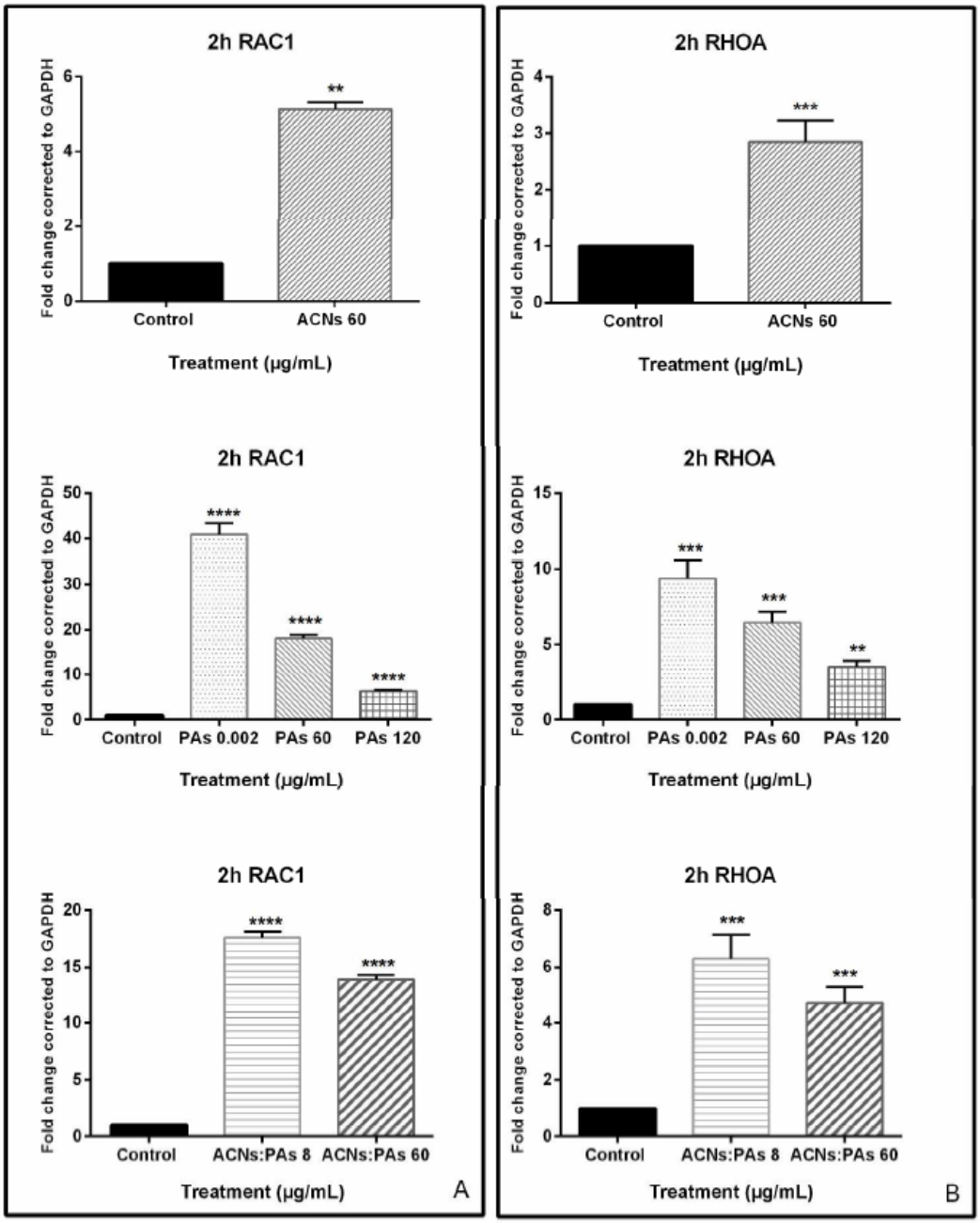
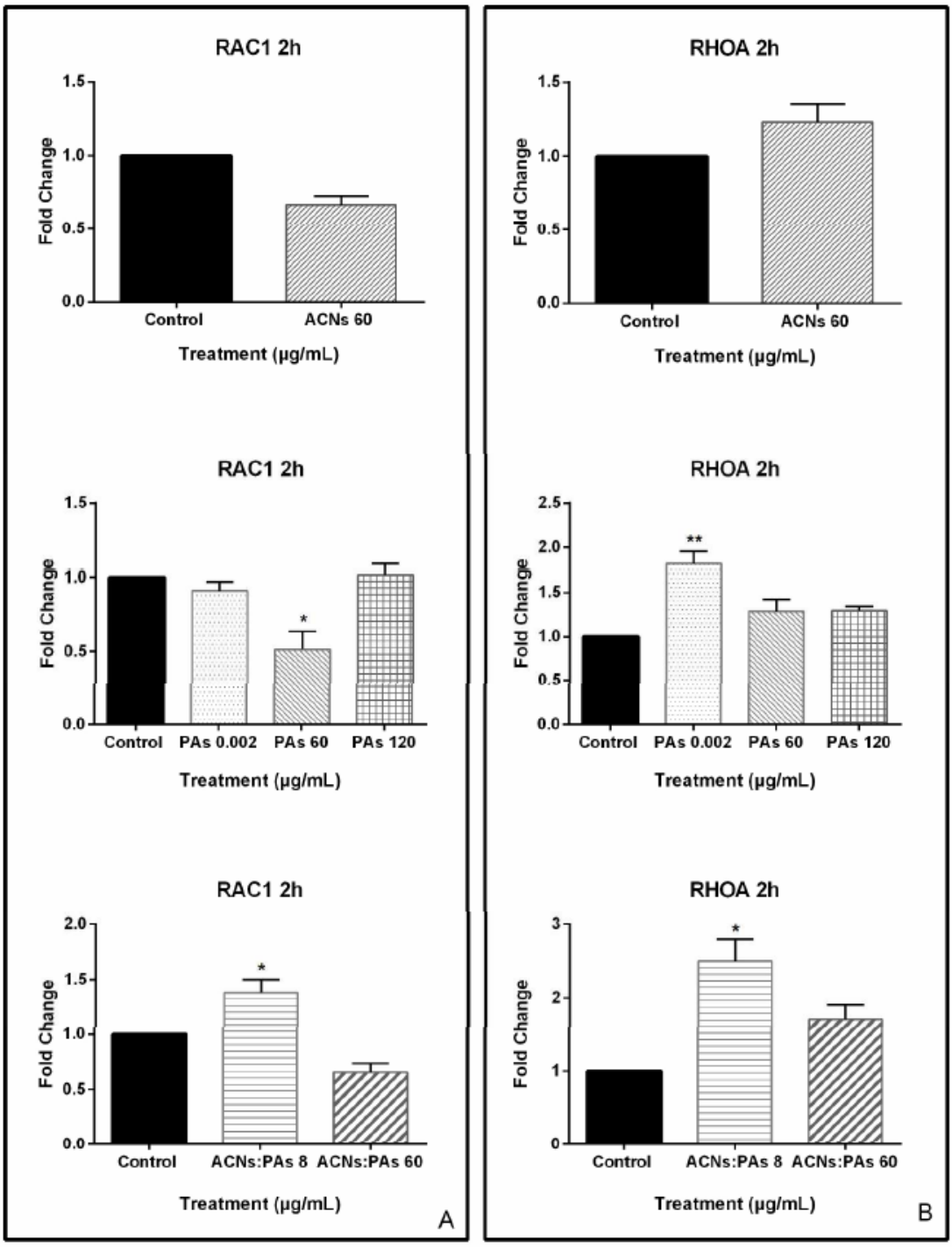

2h RAC1
Fold change corrected to GAPDH
Control
ACNs 60
Treatment (µg/mL)
2h RHOA
Fold change corrected to GAPDH
Control
ACNs 60
Treatment (µg/mL)
2h RAC1
Fold change corrected to GAPDH
Control
PAs 0.002
PAs 60
PAs 120
Treatment (µg/mL)
2h RHOA
Fold change corrected to GAPDH
Control
PAs 0.002
PAs 60
PAs 120
Treatment (µg/mL)
2h RAC1
Fold change corrected to GAPDH
Control
ACNs:PAs 8
ACNs:PAs 60
Treatment (µg/mL)
A
2h RHOA
Fold change corrected to GAPDH
Control
ACNs:PAs 8
ACNs:PAs 60
Treatment (µg/mL)
B

Figure 5


RAC1 2h
Fold Change
1.5
1.0
0.5
0.0
Control
ACNs 60
Treatment (µg/mL)
RHOA 2h
Fold Change
1.5
1.0
0.5
0.0
Control
ACNs 60
Treatment (µg/mL)
RAC1 2h
Fold Change
1.5
1.0
0.5
0.0
*
Control
PAs 0.002
PAs 60
PAs 120
Treatment (µg/mL)
RHOA 2h
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
PAs 0.002
PAs 60
PAs 120
Treatment (µg/mL)
RAC1 2h
Fold Change
2.0
1.5
1.0
0.5
0.0
*
Control
ACNs:PAs 8
ACNs:PAs 60
Treatment (µg/mL)
A
RHOA 2h
Fold Change
3
2
1
0
*
Control
ACNs:PAs 8
ACNs:PAs 60
Treatment (µg/mL)
B