



RESEARCH ARTICLE

Rosmarinic acid from *Mentha arvensis* attenuates Angiotensin II-induced cell death, cellular senescence and mitochondrial depolarization in human umbilical vein endothelial cells

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Abstract

Endothelial dysfunction plays a pivotal role in the progression of cardiovascular diseases and vascular complications associated with diabetes mellitus. Considering the significant mitigating effects that phytochemicals can have on endothelial dysfunction, further research in this area is warranted, forming the basis of this study. This research aims to evaluate the impact of rosmarinic acid (RA), a phenolic compound isolated from *Mentha arvensis*, on experimental endothelial dysfunction induced by Angiotensin II (Ang II). The purity of the compound was verified through HPLC analysis and Human Umbilical Vein Endothelial Cells (HUVECs) were utilized as the *in vitro* model for the investigations. Cytotoxicity of RA was assessed at various concentrations to determine the optimal concentration. RA demonstrated remarkable efficacy in preventing cell death induced by Ang II, increasing viability to 92% after it had been reduced to 53% by Ang II alone. Co-treatment with RA also reduced membrane injury, as evidenced by the LDH leakage assay. The ability of RA to mitigate cellular senescence induced by Ang II was assessed using the X-gal staining method. Analysis of mitochondrial membrane potential through TMRE flow cytometry revealed that groups co-treated with RA exhibited reduced mitochondrial depolarization compared to the Ang II group. This research confirms the protective effect of RA against both cellular and mitochondrial dysfunction induced by Ang II in HUVECs.

Keywords: angiotensin II; cellular senescence; HUVECs; membrane injury; *Mentha arvensis*; rosmarinic acid

Introduction

The mint genus, known as *Mentha*, is a member of the Lamiaceae family and encompasses a total of 42 distinct species, along with 15 hybrids, as well as several subspecies, variations and cultivars (1). There are two well recognized menthol mints that are currently being cultivated: *Mentha x piperita* L. (Hudson), known as peppermint and *Mentha arvensis* L., also known as cornmint and or Japanese mint (2). *Mentha arvensis* L. is a fragrant herb that finds extensive utilization in the fields of medicine, essential oil production and food flavoring (3). Phenolic phytochemicals, derived from plants, are abundant metabolites that are necessary components of both human and animal diets. The Lamiaceae family is potentially rich in beneficial phytochemicals, including phenolic compounds such as Rosmarinic acid (RA) (4). RA, a naturally occurring phenolic molecule, exhibits a wide range of biological properties, including antioxidant, anti-inflammatory, anti-angiogenic, anti-fibrotic, hepatoprotective and anticancer activities (5-8).

Endothelial activation is the change that is characterized by an abnormal pro - inflammatory and pro-thrombotic state of the endothelial cells that line the lumen of blood vessels, which ultimately results in a decrease in the availability of nitric oxide (NO), impairment of vascular tone and other changes collectively referred to as endothelial dysfunctions (9). It has a role in the progression of cardiovascular disorders such as atherosclerosis, hypertension, heart failure and stroke, as well as the vascular complications associated with diabetes mellitus (10). It has been observed that decreased availability of nitric oxide elevates oxidative stress and inflammation, hence raising Renin - Angiotensin System (RAS) activity, particularly the production of Ang II, which exacerbates hypertension by promoting salt retention and arterial stiffness (11, 12).

Given the potential of phytochemicals to exert significant mitigating effects on endothelial dysfunction, there is a pressing need for further research in this field. While RA has been found to protect against myocardial infarction, very little is known about its impact on endothelial dysfunction, which is

the primary contributor to cardiovascular diseases. The focus of this study is to evaluate the effect of RA isolated from *Mentha arvensis*, on experimental endothelial dysfunction induced by Angiotensin II (Ang II).

Materials and Methods

Cell lines

Human Umbilical Vein Endothelial Cells (HUVECs) were initially obtained from HiMedia, India and cultured in Dulbecco's Modified Eagle's Medium (DMEM) from Sigma-Aldrich, USA. The cell line was propagated in 25 cm² tissue culture flasks with DMEM supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine, sodium bicarbonate and an antibiotic solution containing Penicillin, Streptomycin and Amphotericin B. Cultured cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO₂ using a CO₂ incubator.

Chemicals and reagents

All chemicals and reagents used were of analytical grades procured either from Sigma-Aldrich, USA, Merck, Germany, or HiMedia Laboratories Private Limited.

Isolation of rosmarinic acid rich fractions from *Mentha arvensis*

The isolation of RA from *Mentha arvensis* was conducted by the previously established methodology detailed in our laboratory's publication (13).

Estimation of isolated rosmarinic acid using high-performance liquid column chromatography (HPLC)

For the quantification of RA, an Agilent 1260 series High-Performance Liquid Chromatography (HPLC) system (Agilent Technologies, Palo Alto, CA, USA) was employed. The separation was carried out on Zorbax Eclipse Plus (18 columns) with dimensions of 5 µm, 200 mm × 4.6 mm from Agilent and detection was performed at a wavelength of 330 nm. The mobile phase comprised solvent A (0.1% orthophosphoric acid in water, v/v) and solvent B (0.1% orthophosphoric acid in methanol, v/v) in a 50:50 ratio, with a flow rate of 1 mL/min. The column thermostat was maintained at room temperature and the data obtained were analysed using EZchrom software.

Determination of the cytotoxic effect of isolated rosmarinic acid on HUVEC's

The cytotoxicity and non-toxic dosages of RA were assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, following the protocols established by Mosmann (14) and Anuf (15). HUVECs were exposed to varying concentrations of RA ranging from 6.25 to 100 µg mL⁻¹ and incubated overnight. Subsequently, the cells were treated with 30 µg mL⁻¹ of MTT solution prepared from a stock concentration of 5 mg mL⁻¹ in PBS, followed by a 3-hour incubation period. Following incubation, excess dye was removed by PBS washing and the cells were lysed by treatment with 200 µL of dimethyl sulfoxide (DMSO), followed by 30-min incubation at room temperature until a homogenous color was achieved. After centrifugation for 2 min, the absorbance of the resulting solution was measured at 540 nm using a multi-plate reader (Erbachem, Germany),

with DMSO serving as the blank, to determine cell viability and the LD50 value of RA.

In vitro cytoprotective effect of isolated rosmarinic acid on Angiotensin II induced HUVECs

Endothelial dysfunction was initiated by exposing HUVECs to 10 µM Ang II for 1 hr as per modified methods of Qian et al. (16), followed by different concentrations of RA ranging from 6.25 to 50 µg mL⁻¹. Subsequently, the cells were further incubated at 37 °C with 5% CO₂ for 24 hrs and viability was determined as per standard procedures described previously

Lactate dehydrogenase assay

The LDH leakage assay involved utilizing cell-free supernatant obtained from tissue culture plates. In a cuvette, 2.7 mL of potassium phosphate buffer and 0.1 mL of 6mM NADH solutions were added to the cell-free supernatant, thoroughly mixed and then measured for the decrease in optical density at 340 nm using a spectrophotometer maintained at 25 °C. A blank solution for reference was prepared and LDH leakage was determined using an extinction coefficient.

X-GAL staining

The cells were rinsed twice with phosphate-buffered saline (PBS) and then fixed using a solution consisting of 2% formaldehyde and 0.2% glutaraldehyde in PBS for 3-5 minutes at room temperature. Following fixation, they were washed twice with PBS before applying a staining solution comprising 1mg mL⁻¹ X-gal in PBS, 2mM MgCl₂, 150mM NaCl, 5mM potassium ferricyanide, 5mM potassium ferrocyanide and 40mM citric acid/Na phosphate buffer at pH 6.0. The cells were incubated overnight with the staining solution at 37°C. After incubation, they underwent two additional rinses with H₂O and were allowed to air-dry. Subsequently, the cells were observed under an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and the findings were documented through imaging.

Mitochondrial membrane potential by flow cytometry

Cells grown in T25 flasks were allowed to reach 70% confluence. One flask was treated with Ang II, while another flask exposed to Ang II was treated with RA. An untreated control flask was also maintained. After 24 hrs of incubation, cells were detached using trypsin and analyzed by flow cytometry. The cell suspension was collected, resuspended in TMRE (tetramethyl rhodamine ethyl ester) and incubated at room temperature for 5 minutes. Following this incubation period, the samples were loaded onto a flow cytometer (Millipore, USA) and events were recorded after gating, which were then compared with control samples.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism Software 5.01 (GraphPad Software, Inc., San Diego, CA). The results were expressed as the mean ± SD (n = 3) and analyzed using one-way analysis of variance (ANOVA), with P < 0.05 considered statistically significant.

Results

The study aimed to investigate the potential effects of RA on endothelial dysfunction induced by Ang II. RA has been extensively studied for its potent radical scavenging activity, focusing on its antioxidant, neuroprotective, anti-inflammatory and antibacterial properties and associated benefits (17-20). There is limited data available on its specific impact on endothelial dysfunction. Thus, this study sought to address this gap by examining the effects of RA in the context of Ang II-induced endothelial dysfunction, shedding light on its therapeutic potential in this critical area.

Isolation and characterisation of rosmarinic acid

An HPLC analysis was conducted to quantify RA. The concentration of RA was determined to be 24.73 ppm, with a retention time of 6.543 min which was similar to that of standard RA. The results are following our previous findings (13).

In vitro cytotoxic effect of isolated rosmarinic acid on HUVECs

Endothelial cells are a vital constituent of vascular homeostasis, lining the inner layer of blood vessels. They execute pivotal roles in angiogenesis, the process wherein new blood vessels originate from pre-existing ones. Moreover, endothelial cells are significant in several vascular diseases, including atherosclerosis, where their dysfunction contributes to pathogenesis; this lends credence to the efficacy of HUVECs in endothelial research.

The cytotoxic effect of RA was assessed using the MTT assay. HUVECs were treated with varying concentrations of RA and the MTT assay was performed to evaluate cell viability. The results obtained from the MTT assay indicated that RA did not exhibit toxicity within the tested concentration range (Table 1). Furthermore, the half-maximal inhibitory concentration (IC₅₀) of RA was determined to be 196.8 $\mu\text{g/mL}$ using ED₅₀ Plus V0.1 software. This finding suggests that RA is not cytotoxic to HUVECs at the concentrations tested and supports its potential suitability for further investigation in endothelial dysfunction models.

The morphological changes induced by various concentrations of RA were photographed with the aid of an epi-fluorescent microscope (Olympus CKX41) connected to a Pro5 CCD camera (Fig. 1).

In vitro cytoprotective effect of isolated rosmarinic acid on Angiotensin II induced HUVECs

In our study, cell lines were exposed to angiotensin II (Ang II) alone and in combination with RA at varying concentrations. The MTT assay was then employed to evaluate the cytoprotective effect of RA. We observed a notable decrease in cell viability by 53.46% upon exposure to Ang II compared to untreated control cells. However, cells treated with Ang II in conjunction with RA exhibited a significant increase in cell viability, reaching approximately 92.07% at a concentration of 12.5 $\mu\text{g mL}^{-1}$. These results confirm the cytoprotective potential of RA, prompting further investigation into its mechanistic actions.

The percentage of viable cells under different treatment conditions is summarized in Table 2. Morphological changes induced by various concentrations of RA were captured using an epi-fluorescent microscope (Olympus CKX41) equipped with a Pro5 CCD camera (Fig. 2). With RA treatment, we observed a nearly 40% increase in cell viability along with improvements in cell morphology.

Lactate dehydrogenase leakage assay

We studied the effect of RA on cellular membrane integrity in the presence of Ang II and LDH activity measurement in cell culture supernatants was used as an indicator of membrane leakage. Our findings revealed a substantial increase in LDH leakage in cells exposed to Ang II (0.01983 U mL^{-1}) compared to untreated control cells (0.00290 U mL^{-1}). However, co-

Table 1. *In vitro* cytotoxic effect of RA on HUVECs in terms of percentage viability

Concentration of Samples ($\mu\text{g mL}^{-1}$)	Percentage of viability (%)
Control	100
6.25	82
12.5	80
25	79
50	71
100	67

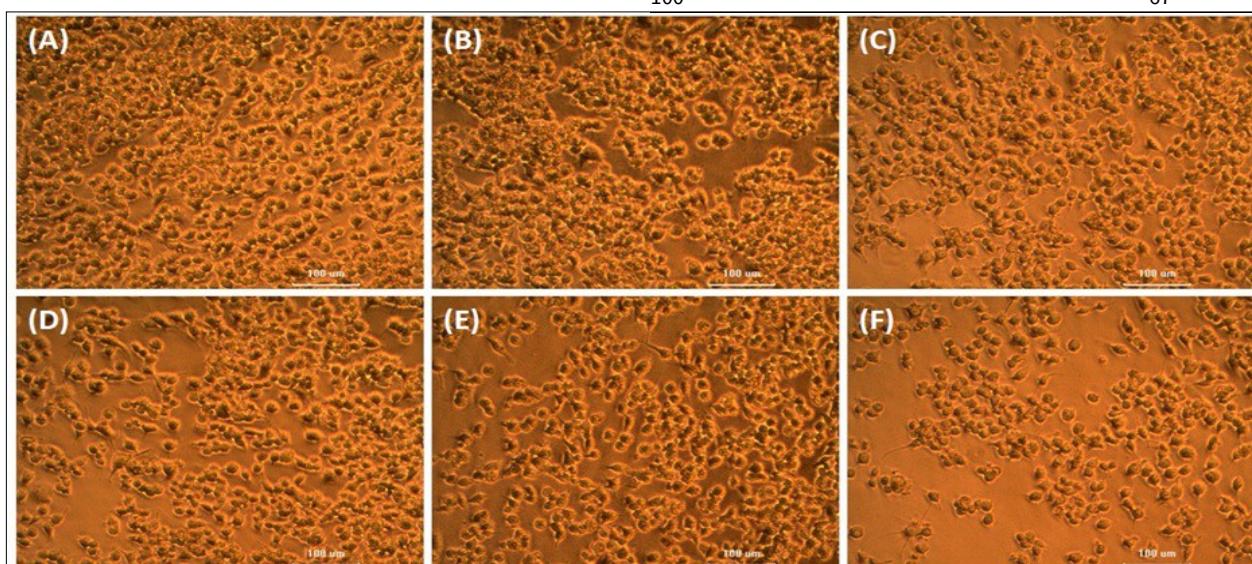


Fig. 1. *In vitro* cytotoxic effect of RA on HUVECs at varying concentrations: (A) Control, (B) 6.25 $\mu\text{g mL}^{-1}$, (C) 12.5 $\mu\text{g mL}^{-1}$, (D) 25 $\mu\text{g mL}^{-1}$, (E) 50 $\mu\text{g mL}^{-1}$, (F) 100 $\mu\text{g mL}^{-1}$.

Table 2. *In vitro* cytoprotective effect of RA on HUVECs in terms of percentage viability

Concentration of samples ($\mu\text{g mL}^{-1}$)	Percentage of viability (%)
Control	100
Angiotensin II	53.46
6.25	75.59
12.5	92.07
25	76.42
50	61.87

administration of RA ($0.00822 \text{ U mL}^{-1}$) led to a significant decrease in LDH leakage compared to cells exposed solely to Ang II (Fig. 3). Specifically, our results demonstrate a reduction of more than 50% in LDH release following treatment with RA, indicating a marked improvement in cellular membrane stability conferred by RA treatment. These findings underscore the protective effect of RA against Ang II-induced membrane damage, further supporting its potential therapeutic utility in mitigating endothelial dysfunction.

Our investigation confirms that cultured HUVECs exposed to Ang II exhibited an increase in blue-stained senescent cells, indicative of cellular senescence. However,

administration of RA significantly reduced the number of senescent cells observed (Fig. 4). These results validate the anti-senescent effect of RA, suggesting its potential to ameliorate endothelial cell senescence induced by Ang II.

Determination of mitochondrial membrane potential by flow cytometry

The mitochondrial membrane potential was evaluated using flow cytometry and the results demonstrate that co-administration of RA significantly alleviated mitochondrial damage induced by Ang II. In the presence of Ang II, the percentage of live cells significantly decreased (24.60%) compared to untreated control cells (86%). However, co-administration of RA (78%) resulted in less mitochondrial damage compared to the Ang II-exposed groups (Fig. 5). Our findings suggest that Ang II treatment notably increased mitochondrial depolarization, leading to a majority of cells becoming depolarized or deceased. Conversely, RA exhibited efficacy in restoring membrane integrity and mitochondrial function.

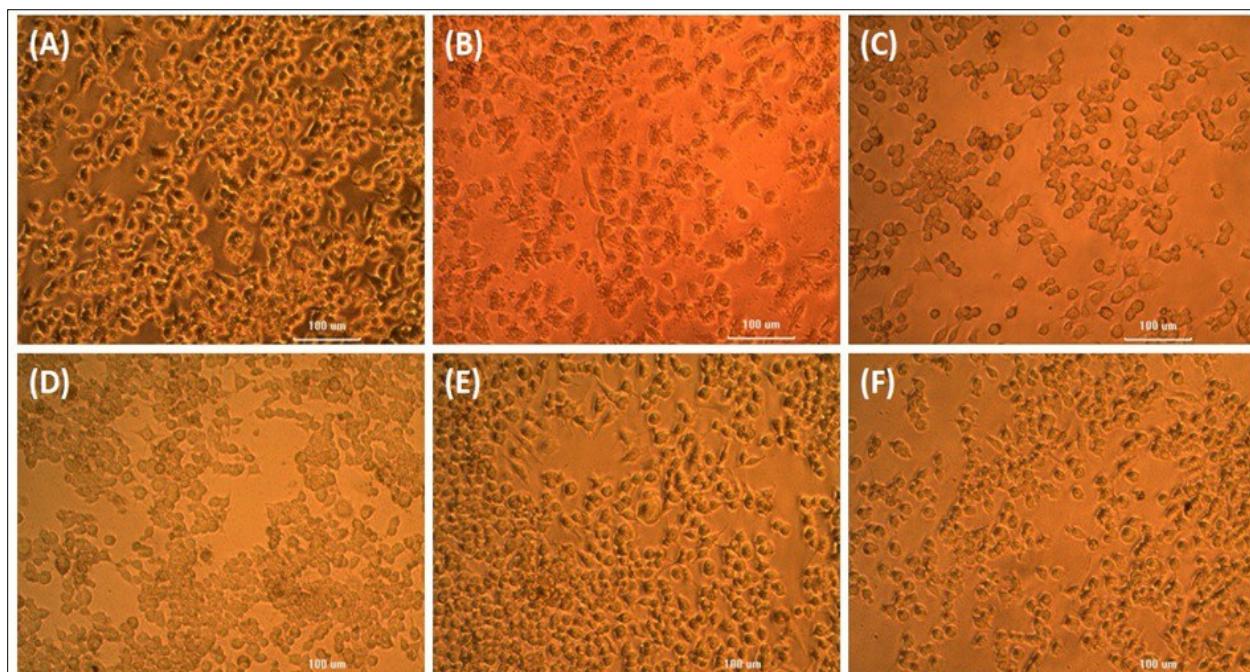


Fig. 2. *In vitro* cytoprotective effect of Rosmarinic acid in terms of % viability on HUVECs cells with varied concentrations. (A) Control (B) Ang II induced and Ang II induced with varied concentrations of Rosmarinic acid (C) 6.25 (D) 12.5 (E) 25 (F) $50 \mu\text{g mL}^{-1}$.

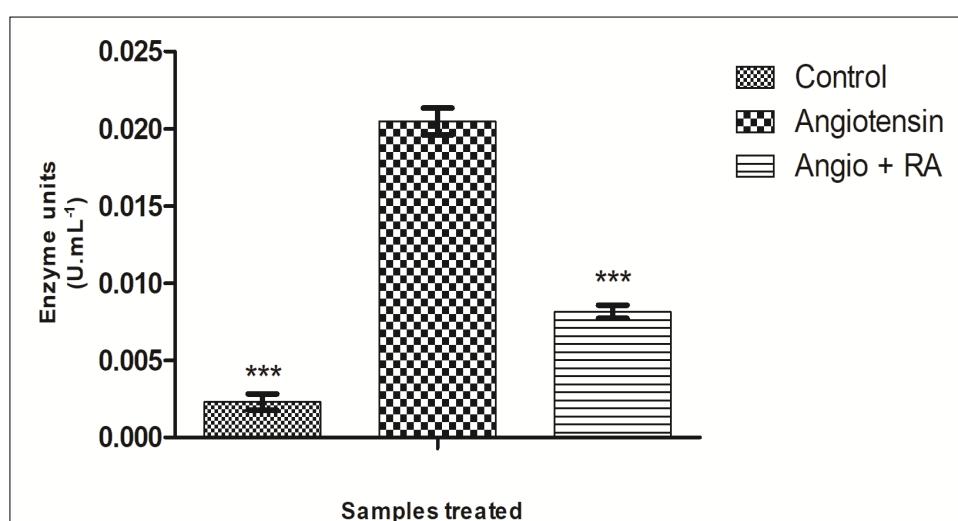


Fig. 3. Graphical representation depicting the lactate dehydrogenase assay, *** $p < 0.001$ vs. control.

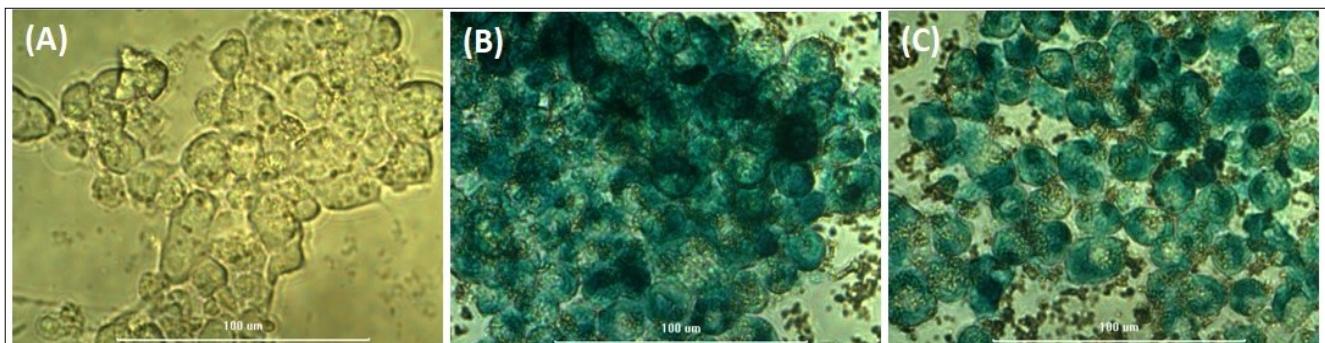


Fig. 4. X-gal staining (A) Untreated control (B) angiotensin II (C) angiotensin II exposed cells co-administrated with RA.

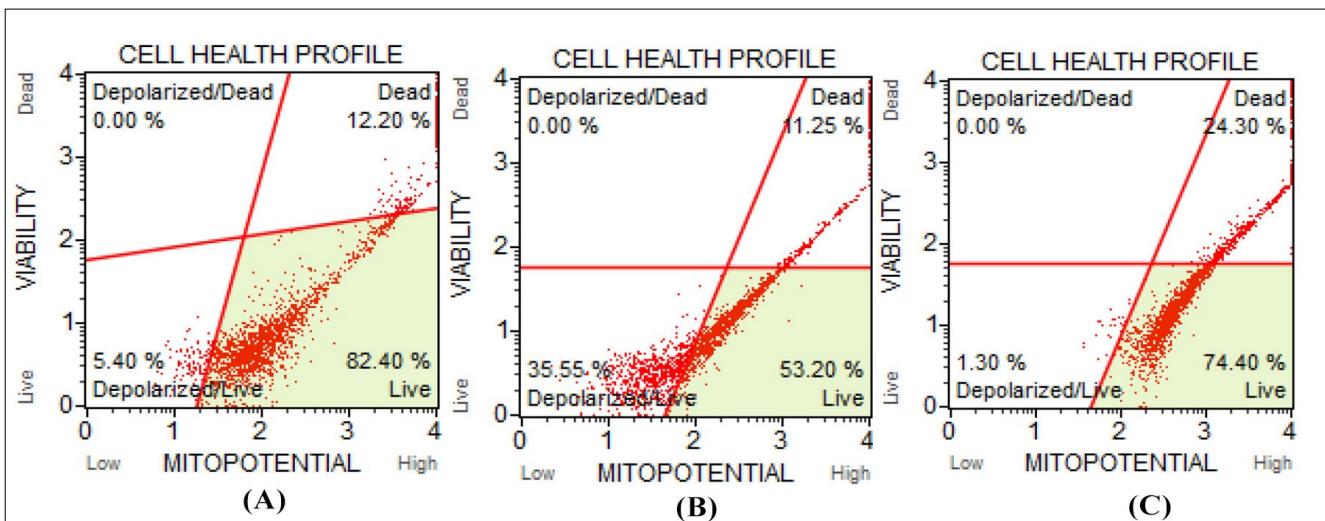


Fig. 5. Flow cytometry performed for (A) control, (B) Ang II-induced group and (C) RA co-administration.

Discussion

The investigation attempted to examine the possible impacts of RA on Ang II-induced endothelial dysfunction. Since data on its precise influence on endothelial dysfunction are scarce, this work aimed to investigate the effects of RA on Ang II-induced endothelial dysfunction, highlighting its therapeutic potential in this significant area.

Following an established technique, rosmarinic acid was isolated and characterized (13). Activation of the renin - angiotensin-aldosterone system (RAAS) is associated with a heightened risk of various cardiovascular pathologies, including glomerulosclerosis, cardiac hypertrophy, atherosclerosis and systemic hypertension. Among its active peptides, Ang II plays a significant role in regulating vascular tone and has been implicated in directly inducing vascular endothelial cell apoptosis through its interaction with the Ang II type 1 receptor (AGTR1) (21). Thus, we utilized angiotensin II (Ang II) as a stimulus to induce endothelial dysfunction.

RA demonstrated no toxicity within the evaluated concentration range and the half-maximal inhibitory concentration (IC_{50}) of RA was also ascertained. The experimental findings validate the cytoprotective potential of RA, underscoring the need for further exploration of its molecular mechanisms. RA therapy resulted in a significant enhancement in cell viability, accompanied by improvements in cell morphology. These data highlight the preventive ability of RA against endothelial dysfunction in HUVECs, similar to the protective effects of vitamin D (22).

Endothelial senescence, characterized by heightened activity, secretary profile, pro-inflammatory state and

aberrant morphological features, has emerged as a significant contributor to various cardiovascular and metabolic disorders (23). Previous review studies have highlighted the presence of senescent endothelial cells on atherosclerotic plaques in the human aorta and coronary arteries (24). A widely recognized marker of cellular senescence is the lysosomal enzyme β - galactosidase (SA - β - gal), typically assessed at pH 6. While β - gal is most active at low pH (pH 4), characteristic of lysosomes, SA- β - gal activity is commonly measured via *in situ* staining using a chromogenic substrate like X-gal, which yields a blue color in the presence of β - galactosidase activity. The study demonstrated the anti-senescence effects of RA and its ability to reduce Ang II -induced endothelial cell senescence.

Endothelial mitochondrial dysfunction is a pivotal factor contributing to endothelial malfunction, particularly in the context of atherosclerosis development. Various risk factors associated with atherosclerosis, such as elevated glucose levels, hypertension, ischemia, hypoxia and diabetes, exacerbate mitochondrial dysfunction in endothelial cells (25). The maintenance of mitochondrial membrane potential ($\Delta\Psi_m$) generated by proton pumps is crucial for energy storage during oxidative phosphorylation. Stable $\Delta\Psi_m$ and ATP levels are essential for cellular viability and prevention of pathological conditions. Additionally, $\Delta\Psi_m$ plays a vital role in mitochondrial homeostasis by selectively eliminating dysfunctional mitochondria and facilitating the transport of ions and proteins necessary for mitochondrial health (26).

Tetra methyl rhodamine ethyl ester (TMRE), a positively charged red-orange dye, permeates cells and

accumulates in active mitochondria due to their relative negative charge. Inactive or depolarized mitochondria exhibit reduced membrane potential and fail to sequester TMRE. RA demonstrated effectiveness in restoring membrane integrity and mitochondrial functionality. The results align with Mailloux's research, which indicated that antioxidants may restore mitochondrial membrane potential, with RA especially helpful in correcting Ang II-induced mitochondrial dysfunction (27). Angiotensin II (Ang II) is reported to play a significant role in promoting mitochondrial membrane depolarization by activating NADPH oxidase, resulting in an increased generation of reactive oxygen species (ROS). These elevated ROS levels cause damage to the mitochondrial membrane potential ($\Delta\psi_m$), impairing mitochondrial function and contributing to endothelial dysfunction. Studies have shown that antioxidants can effectively reduce Ang II-induced oxidative stress and preserve mitochondrial integrity in endothelial cells (28, 29).

Conclusion

Endothelial dysfunction, marked by impaired endothelial cell function, serves as a central contributor to the development of various cardiovascular diseases (CVDs) like atherosclerosis, hypertension and coronary artery disease. The dysfunction in the endothelium leads to diminished bioavailability of NO, heightened oxidative stress, inflammation and irregular regulation of vascular tone, thereby initiating and advancing CVDs. Despite advancements in therapeutic approaches, the clinical burden of CVD remains substantial, prompting the exploration of new treatment options. Novel strategies targeting endothelial dysfunction are increasingly recognized as promising avenues for combating CVDs, aiming to restore endothelial function, alleviate vascular damage and ultimately reduce cardiovascular risks. These emerging therapeutic approaches encompass diverse modalities, including pharmacological agents, lifestyle modifications and innovative biomedical technologies, offering optimism for enhanced management and prevention of CVDs in the future.

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Authors' contributions

RR conducted the design of the study, did a formal analysis and revised the manuscript. AS and VSL performed the investigation and optimization of methodology and performed the statistical analysis. ML and RA participated in the plant collection, data interpretation, draft preparation and adding scientific suggestions. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors declare that there exists no conflict of interest.

Ethical issues: None

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