Citral Protects Human Endothelial Cells against Hydrogen Peroxide-Induced Oxidative Stress

Sitral, İnsan Endotel Hücrelerini Hidrojen Peroksitin Neden Olduğu Oksidatif Strese Karşı Korur

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ABSTRACT

Objectives: Oxidative stress has a major role in endothelial dysfunction. Citral is a monoterpene aldehyde with antioxidant properties. This study aimed to investigate the effect of citral on human umbilical vein endothelial cells (HUVECs) under hydrogen peroxide (H_2O_2)-induced oxidative stress.

Materials and Methods: The cells were treated with citral (0.625-10 µg/ml) for 24h before exposure to H_2O_2 (0.5 mM, 2h). Cell viability was evaluated by 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) assay. The hydroperoxide concentrations and ferric reducing ability of plasma (FRAP) were measured in intra- and extra-cellular fluids.

Results: Pretreatment of HUVECs with citral at the concentrations of 5 and 10 µg/ml significantly enhanced the cell viability in H_2O_2-induced cytotoxicity. It reduced the intra-cellular hydroperoxides level at the concentrations of 5 and 10 µg/ml and the extra-cellular hydroperoxides level at the concentrations of 2.5-10 µg/ml. Pretreatment with citral significantly increased the FRAP value in intra- and extra-cellular fluids at the concentration range of 1.25-10 µg/ml.

Conclusion: Antioxidant and cytoprotective effects were found for citral against oxidative damage induced by H_2O_2 in human endothelial cells. However, more studies in this area are needed to assess its clinical value for prevention and treatment of cardiovascular diseases.

Key words: Citral, HUVECs, oxidative stress, antioxidant, hydrogen peroxide

ÖZ

Amaç: Oksidatif stres endotel disfonksiyonda önemli bir rol içermektedir. Sitral, antioksidan özelliklere sahip olan monoterpen bir aldehittir. Bu çalışma, sitralin, hidrojen peroksit (H202) kaynaklı oksidatif stres altındaki insan göbek ven endotel hücreleri (HUVEC'ler) üzerindeki etkisini araştırma amaçlı yapılmıştır.

Gereç ve Yöntemler: Hücreler, H2O2'ye (0.5 mM, 2 saat) maruz kalmadan önce 24 saat boyunca sitral (0.625-10 µg/ml) ile bırakılıp tedavi edildi. Hücre canlılığı, 3-(4, 5-Dimetiltiazol-2-yl)-2, 5-difenil- tetrazolyum bromür (MTT) tahlili ile denendi ve değerlendirildi. Hidroperoksit yoğunluğu ve plazmadaki ferrik indirgeme kabiliyeti (FRAP) hücre içi ve dışı sıvılarda ölçüldü.

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Bulgular: HUVEC'lerin önceden sitral ile 5 ve 10 µg/ml yoğunluğunda maruz kalması, H2O2 kaynaklı sitotoksisitede hücre canlılığını önemli ölçüde arttırdı. Bu hücre içi hidroperoksit seviyesini 5 ve 10 µg/ml yoğunluğunda ve hücre dışı hidroperoksit seviyesini ise 2.5-10 µg/ml yoğunluğunda düşürdü. Sitrale önceden maruz kalmaları, 1.25-10 µg/ml yoğunluk aralığında hücre içi ve hücre dışı sıvılarda FRAP değerini önemli ölçüde arttırdı.

Sonuç: İnsan endotel hücrelerinde H2O2'nin neden olduğu oksidatif hasara karşı sitral için antioksidan ve sitoprotektif etkiler bulundu. Ancak, kardiyovasküler hastalıkların önlenmesi ve tedavisinde klinik değeri ve yerini değerlendirmek için bu alanda daha fazla çalışma ve deneye ihtiyaç vardır.

INTRODUCTION

Cardiovascular diseases (CVDs) are the most prominent causes of death worldwide.[1] Many studies have confirmed the pivotal role of oxidative stress in the pathogenesis and progression of CVDs. Oxidative stress is a state of overproduction of free radicals and an imbalance between oxidants and antioxidants. Cellular damages and endothelial dysfunction resulted from excessive generation of reactive oxygen species (ROS) have been involved in various CVDs.[2] Superoxide anion, hydroxyl radicals, lipid radicals and hydrogen peroxide (H_2O_2) are examples of ROS in the vascular system. ROS have a physiological role in the controlling cardiovascular homeostasis by mediating of diverse biological responses such as induction of host defense genes, activation of transcription factors, phosphorylation of kinases and mobilization of ion transport systems.[3,4] Beside physiological role, ROS also play an important pathophysiological role in the inflammation, hypertrophy, proliferation, apoptosis, migration, fibrosis, angiogenesis, vascular remodeling and endothelial dysfunction.[5,6]

Natural antioxidants are widely distributed in fruits, vegetables and medicinal plants, produced via the secondary metabolisms and possessing various biological activities.[7,8] In many investigations, herbal antioxidants and bioactive plant constituents have been associated with beneficial therapeutic effects and a reduction in the risk of CVDs.[9]

Citral (3, 7-dimethyl-2, 6-octadienal) is one of the most important natural flavoring compounds, widely used in food, pharmaceutical and cosmetic industries.[10] This monoterpene aldehyde also called lemonal and is a mixture of geranial (trans-citral or citral A) and neral (cis –citral or citral B).[11] Citral is present in several plants with lemon aroma such as lemongrass (*Cymbopogon citratus*) and lemon balm (*Melissa officinalis*).[12,13] This essential oil has revealed antifungal, bactericidal, insecticidal, anticancer, analgesic, anti-inflammatory, anticonvulsant and spasmolytic activities in pharmacological studies.[10,14] Moreover, some beneficial cardiovascular effects due to antioxidant, radical scavenging, anti-inflammatory and vasodilatory properties have been reported for citral.[15,16] The present study aimed to investigate the possible protective effects of citral against oxidative damages induced by H_2O_2 in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Cell culture

HUVECs were maintained in Dulbecco's modified Eagle's medium during a humidified atmosphere of 5% CO_2 at 37°C. The medium was supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml

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streptomycin. Citral (Sigma, Germany) was dissolved in dimethyl sulfoxide 0.8% and diluted with cell culture medium to get different concentrations as per requirement.

Cell viability assay

The viability of HUVECs was determined using 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-diphenyl- tetrazolium bromide (MTT) assay (Bioidea Co., Tehran, Iran) for evaluation of the potential cytotoxicity of citral under normal conditions and also its possible cytoprotective effect against oxidative stress.[17] In Brief, the cell monolayer in exponential growth was harvested and 1.5×10^5 cells/ml was seeded in each well of the 96-well plates. Twenty four hours after plating, HUVECs were treated with 0.625 to 100 µg/ml of citral and incubated for additional 24 h for assessment of the effect of citral on HUVECs proliferation during normal conditions. After washing out with PBS, MTT reagent was added to each well and re-incubated for 3 h. Then dimethyl sulfoxide was used and the absorbance was measured at 570 nm by a microplate reader.

For evaluation of the cytoprotective effect of citral on HUVECs against H_2O_2-induced oxidative stress, the cells were pre-incubated with 0.625 to 10 µg/ml citral for 24 h and then citral was removed from the media and the wells were washed out with PBS. After that the cells were exposed to 0.5 mM H_2O_2 for 2 h. The rest of the experiment was performed as above. The cells without any exposure to the extract or H_2O_2 were considered as negative control. The viability of treated samples was measured according to the following formula and each experiment was tested in triplicate:

Cell viability (%) = (OD test - OD blank/ OD negative control - OD blank) × 100

Hydroperoxides assay

Ferrous ion oxidation by xylenol orange (FOX1) kit (Hakiman Shargh Research Co., Isfahan, Iran) was used for evaluation of the effects of pretreatment with citral on intra- and extra-cellular hydroperoxides level. In this method, hydroperoxides are detected based upon oxidation of reagent Fe^{+2} to Fe^{+3} by oxidizing agents and formation of a color complex through its binding to xylenol orange in an aqueous medium containing sorbitol.[18] Ten µl of supernatant of the cells or the cell lysates after being pretreated with different concentrations of citral and then exposed to H_2O_2 was mixed with 190 µl of FOX-1 reagent. After incubation for 30 min at 40°C, the absorbance was measured at 540 nm using a microplate reader/spectrophotometer. The hydroperoxides content of the samples were estimated as H_2O_2 equivalents using a H_2O_2 standard curve.

Ferric reducing ability of plasma assay

The effects of citral on intra- and extra-cellular ferric reducing ability of plasma (FRAP) was determined by a commercial kit (Hakiman Shargh Research Co., Isfahan, Iran).[19] In this assay, the total antioxidant capacity is estimated based on the reduction of ferric-tripyridyltriazine complex to ferrous form. Briefly, after pretreatment of HUVECs with different concentrations of citral and then exposure to H_2O_2, 10 µl of the supernatant of the cells or the cell lysates was added to 200 µl of FRAP reagent containing tripyridyltriazine/ferric chloride/acetate buffer. The reaction mixture was incubated for 40 min at 40°C and absorbance was read at 570 nm using a microplate

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reader/spectrophotometer. The FRAP value of the samples was calculated using a standard curve of $FeSO_4 \times 7H_2O$ concentrations and were expressed as FeII equivalents.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM). For statistical analysis, one-way analysis of variance (ANOVA) followed by Tukey post-hoc test was performed using SPSS software version 18.0. The p value < 0.05 was considered as significant level.

RESULTS

Effect of citral on HUVECs viability

The potential cytotoxicity of citral on HUVECs was evaluated by MTT assay. There was inhibitory effect on HUVECs proliferation after 24 h exposure to the concentrations of 20-100 µg/ml of citral ($p < 0.001$ compared with control cells) (Figure 1). Therefore, citral was used at the concentration range of 0.625-10 µg/ml for further studies.

Effect of citral on H_2O_2-induced oxidative stress

Figure 2 shows the cytoprotective effect of citral against the oxidative cell death induced by H_2O_2 using MTT assay. H_2O_2 (0.5 mM) produced a significant reduction in HUVECs viability compared with control cells ($p < 0.001$). Pretreatment with citral at the concentrations of 5 and 10 µg/ml significantly enhanced the cell viability in H_2O_2-induced cytotoxicity ($p = 0.005$ and $p < 0.001$, respectively).

Effects of citral on hydroperoxides level

FOX1 assay was performed to detect the effects of citral on intra- and extra-cellular hydroperoxides concentration in HUVECs after exposure to the oxidative stress induced by H_2O_2. A significant increase in hydroperoxides production was observed in the presence of 0.5 mM H_2O_2 as compared to the untreated normal control ($p < 0.001$). Incubation of HUVECs with citral significantly decreased the intra-cellular hydroperoxides level at the concentrations of 5 µg/ml ($p = 0.35$) and 10 µg/ml ($p = 0.001$) compared to the cells treated with H_2O_2 alone (Figure 3A). Citral also caused a significant reduction in the extra-cellular hydroperoxides level at the concentrations of 2.5, 5 and 10 µg/ml ($p = 0.003$, $p < 0.001$ and $p < 0.001$, respectively) (Figure 3B).

Effects of citral on FRAP value

The effect of citral on total antioxidant capacity was evaluated with FRAP assay. Exposure of HUVECs to H_2O_2 resulted in a significant decrease in FRAP value ($p = 0.007$). Pretreatment with citral significantly increased the FRAP value in intra-cellular fluids at the concentrations of 1.25, 2.5, 5 and 10 µg/ml ($p = 0.025$, $p = 0.004$, $p = 0.002$ and $p = 0.003$, respectively) (Figure 4A). It also improved the FRAP value in extra-cellular fluids at the concentrations of 1.25, 2.5, 5 and 10 µg/ml ($p = 0.016$, $p = 0.001$, $p < 0.001$ and $p < 0.001$, respectively) (Figure 4B).

DISCUSSION

Findings of this study showed cytoprotective and antioxidant effects of citral against oxidative stress induced by H_2O_2 in HUVECs. Citral protected the cells against oxidative cell death at the concentrations of 5 and 10 µg/ml. It

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reduced hydroperoxides level and increased FRAP value in both intra- and extra-cellular fluid at different concentration ranges.

H_2O_2 is a stable ROS with capability of readily diffusing through cellular membrane and plays a significant role in the vascular cells signaling such as proliferation, apoptosis and inflammation.[20] H_2O_2 as a trigger of oxidative stress in human endothelial cells has been used in many studies to provide insight into the mechanisms of CVDs development. In this study, the exposure of HUVECs to H_2O_2 caused significant reductions in cell viability and FRAP value and an increase in hydroperoxides level.

Citral, the major constituent of the essential oil of lemon-scented plants has been reported to possess several pharmacological activities.[11] Studies have shown that citral dose- and time-dependently protects some normal cells against distressing stimuli. At higher concentrations, it may show a cell growth inhibitory effect.[21] Our results showed the cytoprotective activity of citral at the concentrations less than 20 µg/ml. Nordin *et al*. reported similar results for the effect of citral on the proliferation of normal spleen cells.[22]

In the present investigation, citral also exhibited antioxidant effects through reducing hydroperoxides level and elevating FRAP value in intra- and extra-cellular fluids. Measurement of ROS provides important data to study the effects of oxidative stress inducers and antioxidant remedies. FOX-1 method is a sensitive assay for detection of hydroperoxides in biological samples.[18] This assess was performed to detect the effects of citral on intra- and extra-cellular hydroperoxides concentration in HUVECs after exposure to the oxidative stress induced by H_2O_2. The effect of citral on total antioxidant capacity was evaluated with FRAP assay. Antioxidants are enzymes or non-enzymatic compounds which involved in the defense mechanisms against oxidative injuries induced by free radicals through preventing ROS creation and scavenging or eliminating ROS.[23] FRAP method is a simple and rapid colorimetric measurement which widely used for screening of non-enzymatic antioxidants.[19] Several studies have reported the antioxidant properties for the phytochemicals in *Citrus* plants.[24] Radical scavenging activity and induction of enzymatic and nonenzymatic cellular antioxidants have been presented for monoterpenes.[14] Cytoprotective and antioxidant effects have been described for *Melissa officinalis* extract as a plant containing high level of citral against H_2O_2-induced oxidative stress in endothelial cells.[25] Vimal and coworkers showed *in vitro* antioxidant effects of some essential oil compounds including citral through evaluation of free radical scavenging, lipid peroxidation, and antioxidant enzymes activities.[16] Bouzenna et al. reported protective effect of citral against aspirin-induced oxidative stress through attenuation of mitogen-activated protein kinases, reduction of malondialdehyde level and modulation of superoxide dismutase and glutathione activities.[26] The protection against high glucose-induced oxidative stress has also been found for citral through inhibiting ROS activated protein kinases signaling pathway in HepG2 cells.[27]

Besides antioxidant properties, some studies has revealed anti-inflammatory effects for citral by suppression of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukins (IL-6 and IL-8), inhibition of inducible endothelial nitric oxide synthase transcription, reduction of p50 nuclear factor-κB levels and suppression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and cyclooxygenase-2 expression.[28-31]

Moreover, the helpful vascular effects including vasodilatory effect likely trough affecting the intracellular calcium concentration and nitric oxide pathway have been found for citral in isolated aorta.[15] Regarding the beneficial cardiovascular activities such as antioxidant, cytoprotective, anti-inflammatory and vasorelaxant effects, citral as a

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natural component could be suggested for prevention of vascular oxidative stress and endothelial dysfunction and consequently prevention of CVDs.

CONCLUSIONS

In conclusion, citral was found to protect HUVECs against oxidative damage induced by H_2O_2 by enhancing total antioxidant capacity and reducing hydroperoxides production. However, more studies in this area are required to evaluate its clinical value for prevention and treatment of CVDs.

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Conflict of Interest: No conflict of interest was declared by the authors.

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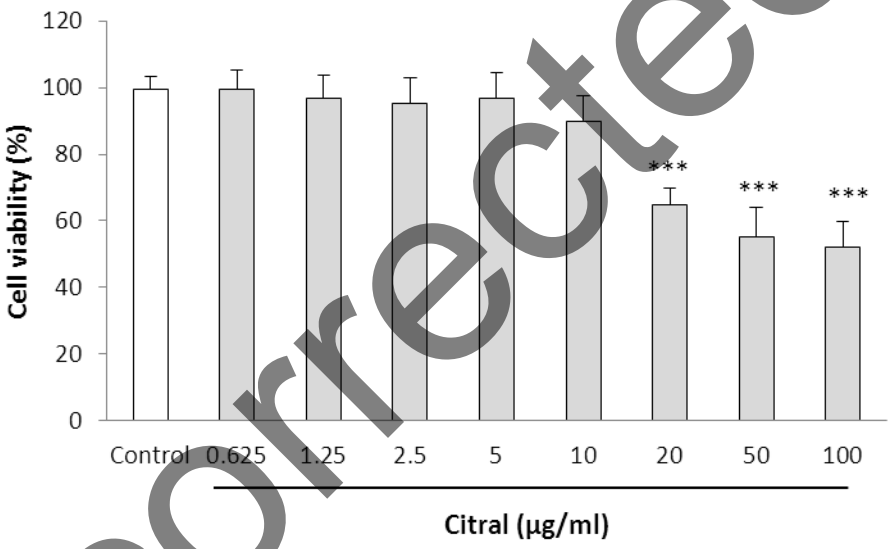

Figure 1. Effect of citral on HUVECs viability determined by MTT assay. Cells were incubated with different concentrations of citral (0.625-100 µg/ml) for 24 h. Values are means ± SEM from three independent experiments in triplicate. $^{***}p < 0.001$ versus control (untreated cells).

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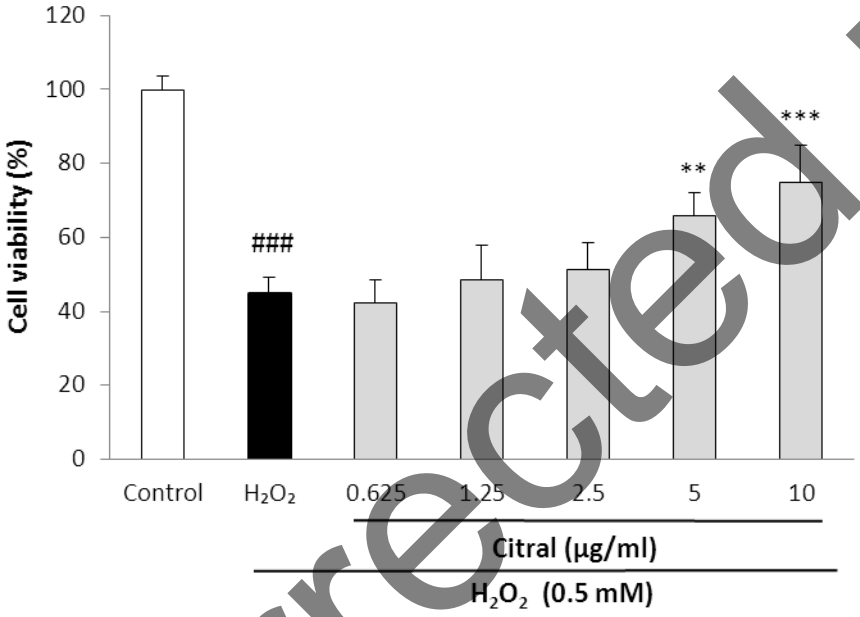

Figure 2. Effect of citral on HUVECs viability in H_2O_2-induced oxidative stress determined by MTT assay. Cells were incubated with H_2O_2 (0.5 mM, 2h) after pretreatment with different concentrations of citral (0.625-10 µg/ml). Values are means ± SEM from three independent experiments in triplicate. $^{###}p < 0.001$ versus control (untreated cells), $^{**}p < 0.01$ and $^{***}p < 0.001$ versus H_2O_2 stimulated cells.

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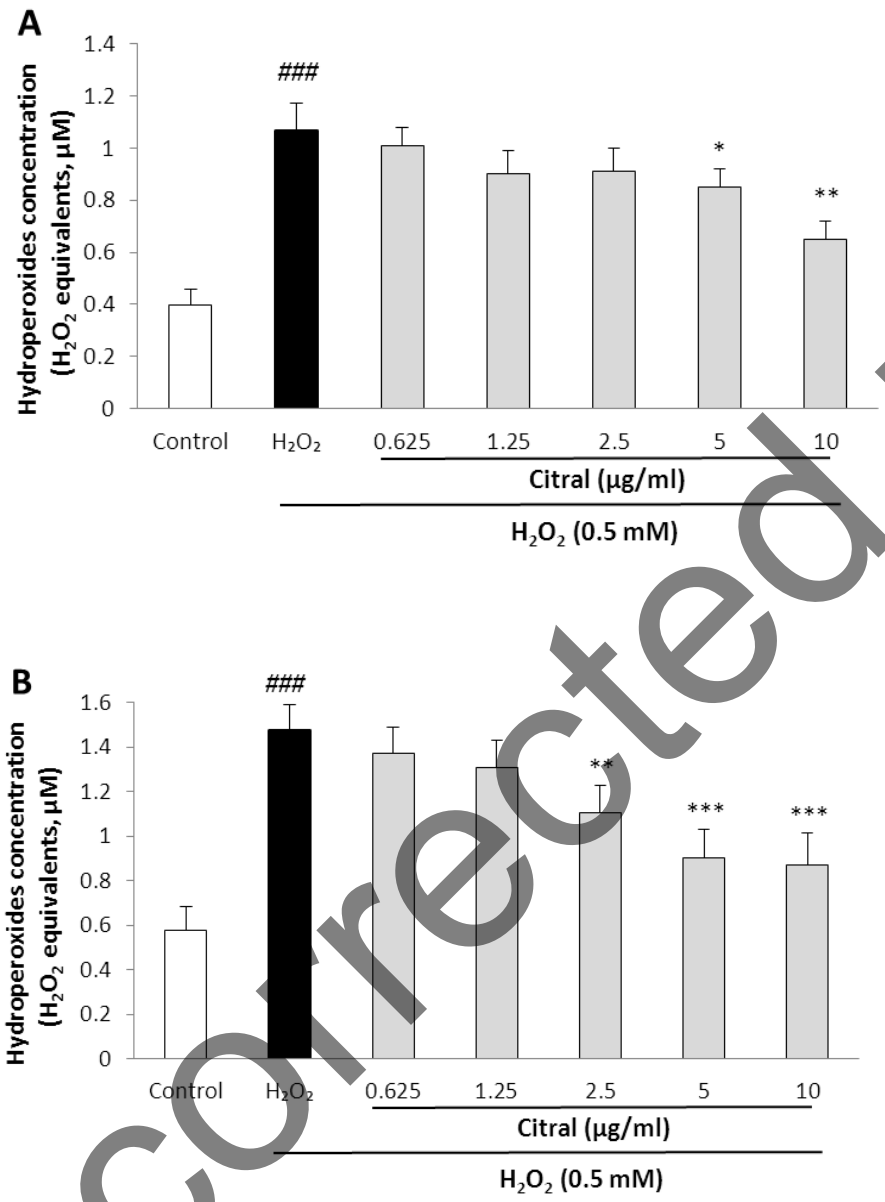

Figure 3. Effect of citral on intra- (A) and extra-cellular (B) hydroperoxides concentration in HUVECs as H_2O_2 equivalents determined by FOX1 method. Cells were incubated with H_2O_2 (0.5 mM, 2h) after pretreatment with different concentrations of citral (0.625-10 µg/ml). Values are means ± SEM from three independent experiments in triplicate. $^{###}p < 0.001$ versus control (untreated cells), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus H_2O_2 stimulated cells.

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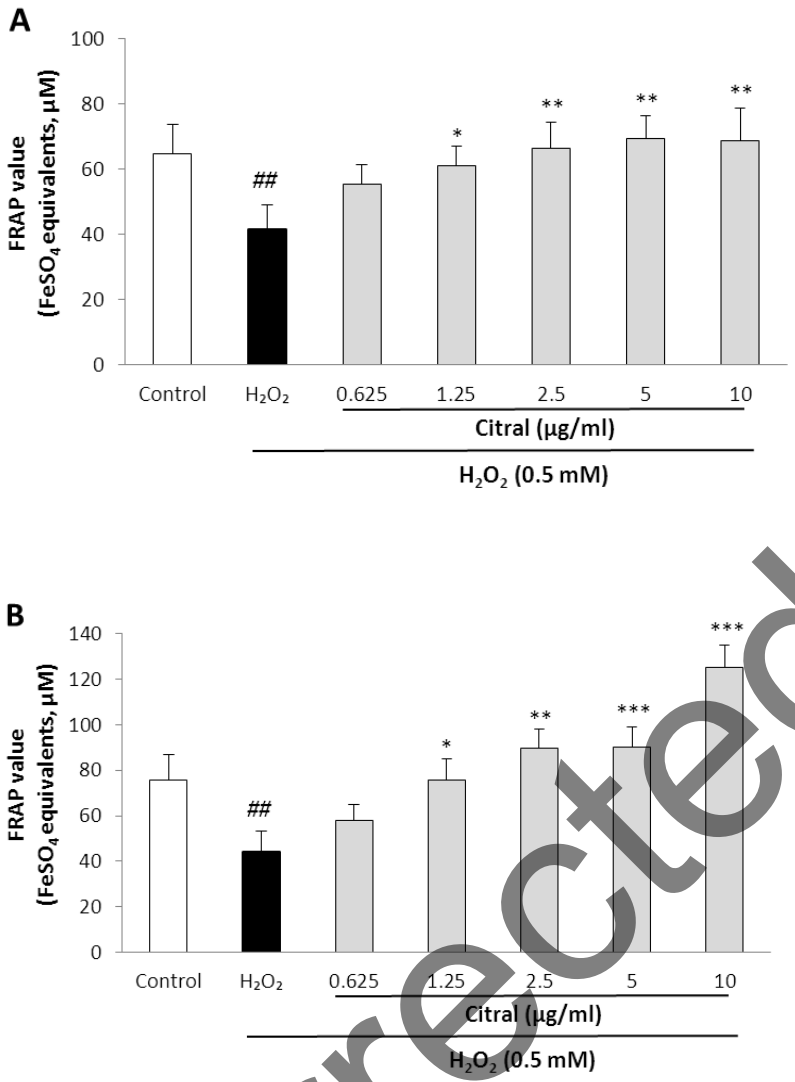

Figure 4. Effect of citral on intra- (A) and extra-cellular (B) FRAP value in HUVECs determined as ferrous sulphate equivalents. Cells were incubated with H_2O_2 (0.5 mM, 2h) after pretreatment with different concentrations of citral (0.625-10 µg/ml). Values are means ± SEM from three independent experiments in triplicate. $^{\#\#}p < 0.01$ versus control (untreated cells), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ versus H_2O_2 stimulated cells.

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