Research in Pharmaceutical Sciences, October 2016; 11(5): 383-389
Received: February 2016
Accepted: April 2016

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Protective effect of *Melissa officinalis* extract against H_2O_2-induced oxidative stress in human vascular endothelial cells

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Abstract

Melissa officinalis L. is a medicinal plant with a large variety of pharmacological effects and traditional applications. This study aimed to evaluate the protective and antioxidant activities of the extract of *M. officinalis* aerial parts on human umbilical vein endothelial cells (HUVECs) under oxidative stress induced by H_2O_2. Cells were incubated with H_2O_2 (0.5 mM, 2 h) after pretreatment with *M. officinalis* extract (25-500 µg/mL). Cell viability was evaluated by 3-(4, 5- Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The concentration of hydroperoxides and ferric reducing antioxidant power (FRAP) were measured in intra- and extra-cellular fluids. Pretreatment of HUVECs with *M. officinalis* extract at the concentrations of 100-500 µg/mL improved the cell viability after exposure to H_2O_2 significantly. It also decreased hydroperoxides concentration and increased FRAP value in both intra- and extra-cellular fluids. The results revealed antioxidant and cytoprotective effects of *M. officinalis* against H_2O_2-induced oxidative stress in HUVECs. Due to the valuable antioxidant activity, this plant extract may have potential benefits for the prevention of cardiovascular diseases associated with oxidative stress.

Keywords: *Melissa officinalis* L.; HUVECs; Oxidative stress; Antioxidant

INTRODUCTION

Oxidative stress plays a great role in the pathophysiological mechanisms involved in cardiovascular diseases. Increased production of free radicals and oxidative cellular damages has been implicated in various cardiovascular diseases such as atherosclerosis, high blood pressure, heart failure, diabetes and hypercholesterolemia (1). Oxidative stress is mainly caused by the production of large amounts of reactive oxygen species (ROS) and reduction of antioxidant capacity. Superoxide anion, hydroxyl radicals, lipid radicals and hydrogen peroxide (H_2O_2) are the examples of ROS. In the vasculature, ROS cause inactivation of nitric oxide, oxidation of low density lipoprotein (LDL), inflammation and impairment of endothelial function associated with hypertension and atherosclerosis (2-4).

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Antioxidants are substances that protect endothelial cells against oxidative toxicity by preventing ROS formation, neutralizing the free radicals and inhibiting oxidation reactions (5-7). A growing body of evidence has shown a relation between supplementation with antioxidants and reduction in morbidity and mortality from cardiovascular diseases. Antioxidants may improve endothelial function through prevention from lipid peroxidation in LDL, enhancement of endothelium-dependent vasodilatory responses and modulation of endothelium-leukocyte interactions (8-10).

Melissa officinalis L. (lemon balm) is a traditional herbal medicine with a large variety of effects such as spasmolytic, analgesic,

Access this article online	
	Website: http://rps.mui.ac.ir
	DOI: 10.4103/1735-5362.192488

hypnotic, hepatoprotective, antibacterial, anticancer, antioxidant and antiviral activities (11-15). Lemon balm grows in central and southern Europe, North America, and in Asia Minor. In Iranian traditional medicine, it is used to treat a wide range of diseases including fever, headaches, influenza, bloating, indigestion, colic, nausea, anemia, dizziness, fainting, weakness, asthma, bronchitis, amenorrhea, heart failure, arrhythmia, insomnia, epilepsy, depression, neurological disorders, wounds and injuries (16,17). Lemon balm contains many antioxidant compounds including flavonoids such as naringin and hesperidin and also hydroxycinnamic acid derivatives such as rosmarinic acid, m-coumaric acid and caffeic acid (18,19).

In this study, protective effect of hydroalcoholic extract obtained from the aerial parts of *M. officinalis* was investigated in human umbilical vein endothelial cells (HUVECs) under oxidative stress induced by H_2O_2. To assure the safety of *M. officinalis* extract on HUVECs, the toxicity of this herbal extract was also evaluated.

MATERIALS AND METHODS

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) was obtained from GIBCO Life Technologies (Paisley, Scotland). Fetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit was purchased from Bioidea Co. (Tehran, Iran). The assay kits for measurement of hydroperoxides and ferric reducing antioxidant power (FRAP) were purchased from Hakiman Shargh Research Co. (Isfahan, Iran). All other chemicals were from Merck Co., Germany.

Plant material and preparation of extract

The aerial parts of *M. officinalis* were collected from Hasan Abad Jarghooyeh in the Isfahan Province during May 2014. After authentication, a voucher specimen (No. 3395) was deposited at the Herbarium of the School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences. For preparation of hydroalcoholic extract, the air-dried aerial parts of *M. officinalis* were powdered and extracted three times with ethanol:water (70:30) using maceration process at room temperature for 72 h. After filtration and evaporation of solvent under reduced pressure using a rotary evaporator, the obtained extract was freeze-dried and the residue was stored at -20 ºC. The yield of the plant extract was 16.8 % (w/w). The extract was dissolved in dimethyl sulfoxide (DMSO) 0.8% and diluted with cell culture medium to get different concentrations as per requirement.

Determination of total phenolic content

The total phenolic content of *M. officinalis* extract was estimated using Folin-Ciocalteu assay. Briefly, the plant samples were mixed with sodium bicarbonate (20%) and diluted Folin's reagent. After 2 h, the absorbance was measured at 765 nm. Each experiment was assayed in triplicate. The total phenolic content was estimated using a standard curve obtained from different concentrations of gallic acid as the standard reference (20).

Cell culture

Human umbilical vein endothelial cells were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Iran, Tehran,). The cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cultures were maintained under normal culture condition (5% CO_2 and 95% humidified air at 37 °C).

Cell viability evaluation

MTT assay was used to determine the effect of *M. officinalis* extract on viability of HUVECs. This assay was based on the reduction of tetrazolium salt by mitochondrial dehydrogenases of viable cells and performed for evaluation of both probable cytotoxic and cytoprotective effects of *M. officinalis* extract in this research (21).

At first, the probable cytotoxicity of the extract (25 to 1000 µg/mL) on normal HUVECs was assessed using MTT method. Briefly, at the end of 24 h incubation period, HUVECs were treated with different concentrations of freshly prepared *M. officinalis* extract and incubated for an

additional 24 h at 37 °C. After washing out with phosphate buffered saline (PBS), new medium containing MTT reagent was added to each well and incubated for 3 h at 37 °C. MTT reaction with living cells creates insoluble foramazan crystals with purple color. After dissolution of foramazan crystals by adding DMSO, the absorbance was measured at 570 nm by a microplate reader (BioTek Instruments, PowerWave XS, Wincoski, USA).

The cytoprotective effect of *M. officinalis* extract on HUVECs was also evaluated under oxidative stress induced by hydrogen peroxide. The cells were pre-incubated with the extract (25 to 500 µg/mL) or vitamin C (100 µg/mL as the standard reference) for 24 h at 37 °C. After removal of the medium and washing out with PBS, HUVECs were exposed to H_2O_2 0.5 mM for 2 h. The rest of the experiment was completed as above. The cells without any exposure to the extract or H_2O_2 were considered as negative control. The cells with exposure to vitamin C were used as the positive control. The viability of treated samples was assessed by comparison of the absorbance of different concentrations of the samples with negative control according to the following formula and each experiment was tested in triplicate (21):

$$Cell\ viability\ (\%) = \frac{OD\ test\ -\ OD\ blank}{OD\ negative\ control\ -\ OD\ blank} \times 100$$

Measurement of extra- and intra-cellular hydroperoxides concentration

The effects of *M. officinalis* extract on intra- and extra-cellular level of hydroperoxides were evaluated based on the ferrous ion oxidation by xylenol orange reagent (FOX-1) (22). The FOX-1 reagent containing ammonium ferric sulfate in aqueous medium with sorbitol was prepared according to the instructions of the kit manufacturer.

The HUVECs were pretreated with different concentrations of the extract and exposed to the H_2O_2. Then 10 µL of supernatant of the cells or the cell lysates from each well was mixed with 190 µL of FOX-1 reagent. After incubation for 30 min at 40 °C, absorbances of the samples were determined using a microplate reader/spectrophotometer at 540 nm. The hydroperoxides content of the samples were estimated as H_2O_2 equivalents from a H_2O_2 standard curve.

Measurement of cell-free and intra- and extra-cellular ferric reducing antioxidant power

The effects of *M. officinalis* extract on intra- and extra-cellular FRAP was determined by Benzie and Strain method (23). This method measures the total antioxidant capacity based on the reduction of ferric-tripyridyltriazine (TPTZ) complex to ferrous form by spectrophotometric assay.

After pretreatment of HUVECs with different concentrations of *M. officinalis* extract, the cells were exposed to oxidative stress by H_2O_2.

The FRAP reagent containing TPTZ/ferric chloride/acetate buffer was freshly prepared according to the instructions of the kit manufacturer. For each well, 10 µL of the supernatant of the cells or the cell lysates was added to 200 µL of FRAP reagent.

The FRAP level was also evaluated on samples without cells. These samples contained different concentrations of *M. officinalis* extract. The reaction mixture was incubated for 40 min at 40 °C and absorbance was measured at 570 nm using a microplate reader/spectrophotometer.

The FRAP value of samples was calculated using a standard curve of $FeSO_4.7H_2O$.

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) in triplicate experiments. Statistical analysis was done by one-way analysis of variance (ANOVA) followed by Tukey post-hoc test (SPSS software version 16.0). *P* value < 0.05 was considered as the criteria for significant differences.

RESULTS

Total phenolic content

The total phenolic content of the hydroalcoholic extract of *M. officinalis* was estimated as 28.84 ± 4.04 mg gallic acid equivalent/g of the dried aerial parts of the plant.

Effect of M. officinalis extract on human umbilical vein endothelial cells viability

In order to evaluate the probable cytotoxicity of *M. officinalis* extract on HUVECs, the cell viability was measured by MTT assay. There was no inhibitory effect on HUVECs viability after exposure to *M. officinalis* extract at the concentrations of 25 - 500 µg/mL for 24 h. However, the concentration of 1000 µg/mL of the extract significantly decreased the HUVECs viability (Fig. 1).

Cytoprotective effect of M. officinalis extract against H_2O_2-induced oxidative stress

Fig. 2 shows the cytoprotective effect of *M. officinalis* extract against the oxidative damage induced by H_2O_2 using the MTT method.

The exposure of HUVECs to 0.5 mM H_2O_2 for 2 h remarkably reduced cell viability ($P < 0.001$).

Pretreatment of HUVECs with *M. officinalis* extract at the concentrations of 100-500 µg/mL significantly decreased the cell death resulted from the exposure to H_2O_2. The protective effect was not observed at concentrations of 25 and 50 µg/mL of the extract.

Effects of M. officinalis extract on intra- and extra-cellular concentration of hydroperoxides

The effects of *M. officinalis* extract on intra- and extra-cellular hydroperoxides concentration estimated as H_2O_2 equivalents by FOX1 method are shown in Fig. 3. The levels of hydroperoxides were markedly reduced in intra- and extra-cellular fluids after pretreatment of the HUVECs with *M. officinalis* extract at the concentrations of 100-500 µg/mL compared with the control group.

Effects of M. officinalis extract on cell-free and intra- and extra-cellular ferric reducing antioxidant power value

In cell-free assay, the FRAP value of the *M. officinalis* extract which was expressed as equivalence of ferrous sulphate showed increasing trend in total antioxidant capacity with the extract concentrations (Fig. 4). In cell-based assay, the FRAP value was markedly increased after pre-treatment with the extract at the concentration range of 100-500 µg/mL in intra-cellular fluids compared with the control group. Pre-incubation with the extract at the concentrations of 50 - 500 µg/mL also increased the total antioxidant capacity in extra-cellular fluids in a concentration-dependent manner (Fig. 5).

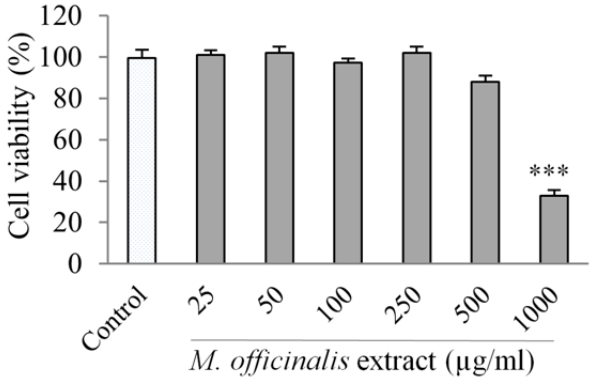

Fig. 1. Effect of *M. officinalis* extract on human umbilical vein endothelial cell viability determined by MTT assay. Cells were incubated with different concentrations of the extract (25-1000 µg/mL) for 24 h. Values are the means ± SEM of three independent experiments. $^{***}P < 0.001$ versus control (untreated cells).

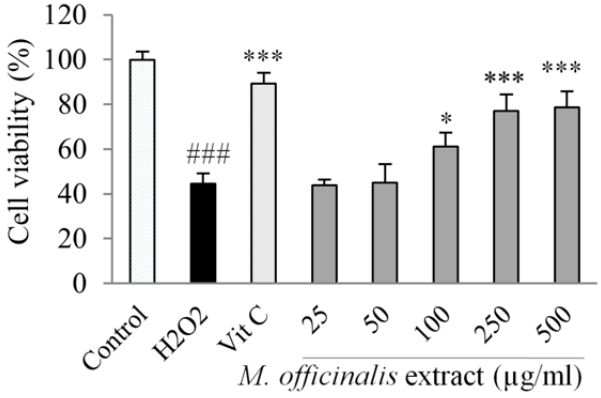

Fig. 2. Effect of *M. officinalis* extract on human umbilical vein endothelial cells viability in H_2O_2-induced oxidative stress. Cells were incubated with H_2O_2 (0.5 mM, for 2 h) after pretreatment with the extract (25-500 µg/mL) or vitamin C (100 µg/mL). The cell viability was determined by the MTT assay. Values are the means ± SEM of three independent experiments. $^{###}P < 0.001$ versus control (untreated cells), $^{*}P < 0.05$ and $^{***}P < 0.001$ versus H_2O_2 stimulated cells.

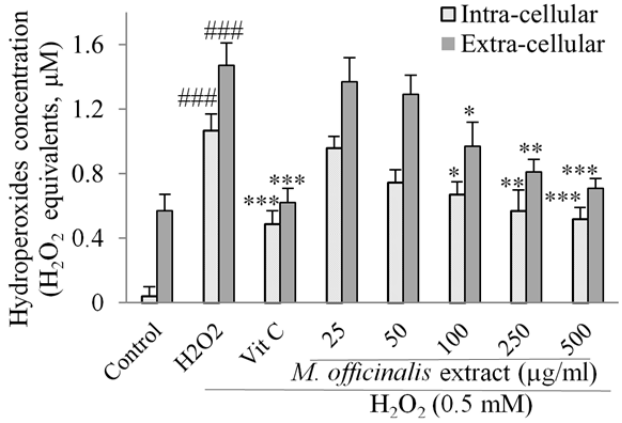

Fig. 3. Effect of *M. officinalis* extract on intra- and extra-cellular concentration of hydroperoxides in human umbilical vein endothelial cells measured as H_2O_2 equivalents determined by ferrous ion oxidation by xylenol orange reagent method. Cells were incubated with H_2O_2 (0.5 mM, for 2 h) after pretreatment with the extract (25-500 µg/mL) or vitamin C (100 µg/mL). Values are the means ± SEM of three independent. $^{###}P < 0.001$ versus control (untreated cells), $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus H_2O_2 stimulated cells.

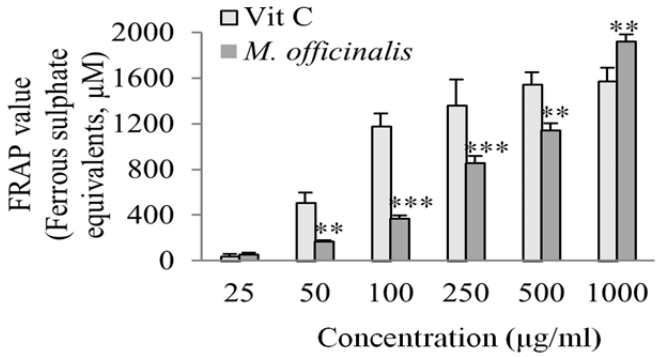

Fig. 4. Ferric reducing antioxidant power values of different concentrations of *M. officinalis* extract and vitamin C (25-1000 µg/mL) determined as ferrous sulphate equivalents. Values are the means ± SEM of three independent experiments. $^{**}P<0.01$ and $^{***}P<0.001$ versus vitamin C control group at the same concentration.

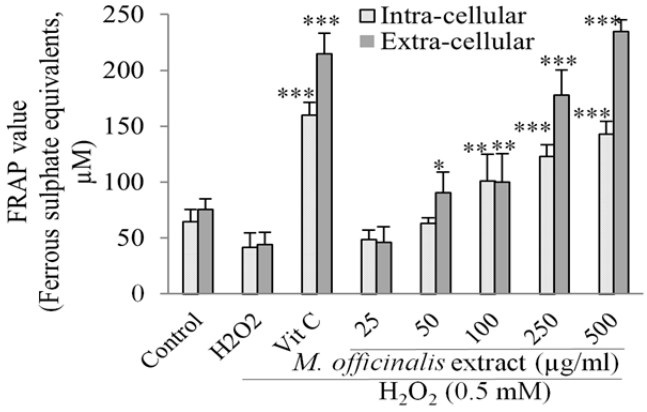

Fig. 5. Effect of *M. officinalis* extract on intra- and extra-cellular ferric reducing antioxidant power value in human umbilical vein endothelial cells determined as ferrous sulphate equivalents. Cells were incubated with H_2O_2 (0.5 mM, for 2 h) after pretreatment with the extract (25-1000 µg/mL) or vitamin C (100 µg/mL). Values are the means ± SEM of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus H_2O_2 stimulated cells.

DISCUSSION

This investigation explored the antioxidant effect of *M. officinalis* hydroalcoholic extract as well as its cytoprotective effect against H_2O_2-induced toxicity in HUVECs. The plant extract protected the cells against oxidative stress at the concentrations of 100-500 µg/mL. The extract decreased concentration of hydroperoxides in intra- and extra-cellular fluids at the concentration range of 100-500 µg/mL and increased FRAP value in intra-cellular fluid at the concentrations of 100-500 µg/mL and in extra-cellular fluid at the concentrations of 50-500 µg/mL.

The role of endothelial cells in the regulation of physiological functions of vascular system has been elucidated in many investigations and studies as well as the role of oxidative stress in the impairment of endothelium function (1). In the present study, HUVEC as a normal cell type with good homogeneity, rapid expansion property and well-characterized surface markers was used in a model of oxidative stress on endothelial cells (24).

We also used FRAP assay for evaluation of antioxidant effects of *M. officinalis* extract in the present investigation. This assay is a simple, inexpensive and reproducible method for measuring antioxidant power in both in vitro and biological fluids (23).

M. officinalis has earlier been revealed to have antioxidant activities (11,18,19). However, the present study was planned to confirm this property at the cellular level. The detected cytoprotective effect against oxidative injury of HUVECs is possibly due to its various antioxidant phytochemicals. Rosmarinic acid has been identified as the major component of *M. officinalis* extract (18). This polyphenolic compound is a caffeic acid derivative with favorable cardiovascular effects such as antioxidant, anti-inflammatory, anti-apoptotic, cyto- and cardio-protective activities (25). Rosmarinic acid is a superoxide scavenger which can inhibit LDL oxidation in human aortic endothelial cells (26). The structure-activity relationship studies have shown the role of an ortho dihydroxyphenyl group and the conjugated double bond in the C3 carbon chain in its scavenging activity (27). The antioxidant effect of rosmarinic acid also contributes in its anti-angiogenesis activity through reducing the expression of H_2O_2-dependent vascular endothelial growth factor, decreasing release of IL-8 from endothelial cells and inhibiting the proliferation, migration, adhesion, and tube formation of HUVECs (27).

Caffeic acid, another antioxidant phenolic compound in the extract of *M. officinalis,* has potential to reduce the risk of cardiovascular disorders by inhibiting the production of ROS (28). It can improve resistance of the cells against oxidative stress by inhibition of lipid peroxidation and reduction of glutathione depletion (29). Hesperidin is another phenolic constituent of *M. officinalis* with cytoprotective effect which has increased the cell viability by providing powerful antioxidant protection against paraquat and H_2O_2-induced oxidative stress (30). The major components of the essential oil of the lemon balm leaf, citronellal and citral are also antioxidant compounds with superoxide scavenging activity (31).

CONCLUSION

In conclusion, the results of this study revealed that hydroalcoholic extract of *M. officinalis* aerial parts has the potential to protect HUVECs under oxidative stress induced by H_2O_2. Due to the presence of various antioxidant phytochemicals, the plant extract may have potential benefits in human health through preventing harmful oxidative injuries.

ACKNOWLEDGMENTS

This research as a project No. 393659 has been financially supported by Vice Chancellor of Research of Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

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