The *in vitro* effect of *Melissa officinalis* aqueous extract on aortic reactivity in rats with subchronic diabetes

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A B S T R A C T

Background and Objective: Vascular abnormality and dysfunction plays an important role in the pathogenesis of vascular disease in diabetic state. In this study, we aimed to investigate whether an *in vitro* exposure of endothelium-intact aortic rings to *Melissa officinalis* (lemon balm, MO) aqueous extract could have a beneficial effect in rats with subchronic diabetes.

Materials and Methods: Diabetes was induced with a single dose of intraperitoneal injection of streptozotocin (STZ, 60 mg/kg). After one month, reactivity of isolated endothelium-intact aortic rings to KCl and phenylephrine (PE) in the absence and presence of blockers of nitric oxide synthase (L-NAME) and prostaglandin synthesis pathway (indomethacin) in addition to the role of extracellular calcium was evaluated in the presence of MO extract in a cumulative manner.

Results: After 1 month, addition of MO extract (at a concentration range of 0.2-1 mg/ml) caused a significant concentration-dependent relaxation of phenylephrine (PE) and not KCl-preconstricted rings in both control and diabetic groups with a significant inter-group difference of $p<0.05$-0.01 for the former group. Furthermore, both L-NAME (100 μM) and not indomethacin (10 μM) significantly diminished the vasorelaxant responses following MO addition. Meanwhile, there was no significant difference between the groups in the absence of extracellular calcium.

Conclusion: These findings show that MO aqueous extract could relax the PE-preconstricted rings of aorta in STZ-diabetic rats through a nitric oxide pathway and prostaglandin pathway does not have a significant role and this extract could not affect the release of calcium from intracellular stores.

1. Introduction

Diabetes mellitus (DM) leads to severe and debilitating cardiovascular complications, and heart disability, diseases and failure remain the major causes of death in diabetic patients. Given the increasing tide of obesity and diabetes worldwide, the clinical burden of diabetes-induced cardiovascular disorders is reaching epidemic proportions. DM also worsens early and late outcomes in acute coronary disorders. Meanwhile, DM itself is a prothrombotic condition, associated with inflammation, altered innate immunity, and impaired endothelial function. In type 1 DM, the main pathogenic mechanism seems to be the destruction of pancreatic β-cells (1).

Thus, urgent measures are warranted to stem the tide of diabetes which entails new prevention and treatment tools (2). Traditional strategies for controlling the cardiovascular complications of diabetes primarily target a cluster of

well-defined risk factors, such as hyperglycemia, lipid disorders and hypertension (3). Considering the great developments in human knowledge about diabetes mellitus diversity and knowing that it is one of the major health, social and economic problems in the world, a need for finding effective compounds, with fewer side effects, to treat diabetes and its complications has been arisen. Although medicinal herbs and their derivatives have been used as a remedy for diabetes mellitus for a long time, their certain effectiveness has not yet been proven by any valid research (4). Medicinal plants and their derived forms (extracts, syrups, etc.) have been the basis of medical therapy for centuries. Traditionally used in the treatment of several human disorders, their pharmacological and therapeutic properties are attributed to various chemical constituents isolated from their crude extracts. However, their correct use requires the manipulation of plants selected for their efficacy and safety, based either on folk tradition or scientific validation (5). Originally native to the east Mediterranean region and west Asia, *Melissa officinalis* (L.) (Lamiaceae) (lemon balm) is a plant with medicinal efficacy and is a well-known medicinal herb that has been used for a long time and has been suggested to be effective in the treatment of multiple conditions. Aqueous and alcoholic extracts from the aerial part of *Melissa officinalis* (MO) are traditionally used in the treatment of fevers and colds, indigestion associated with nervous tension, hyperthyroidism, depression, mild insomnia, epilepsy, headaches, toothaches, and so on. Furthermore, its antioxidant activity has been described. Phytochemical studies carried out with MO have demonstrated the numerous constituents, viz., polyphenolic compounds (rosmarinic acid, caffeic acid and protocatechuic acid), essencial oils (citral), monotherpenoid aldehides, sesquiterpenes, flavonoids (luteolin) and tannins. Pharmacological investigation concerning its essential oil has revealed that, besides this being an efficient antibacterial and antifungal agent (5, 6). Until now, endothelium-dependent induction of vasorelaxation by MO in rat isolated thoracic aorta has been shown (7). Therefore, in this study, we aimed to investigate whether an in vitro exposure of endothelium-intact aortic rings to MO aqueous extract could have a beneficial effect in rats with subchronic diabetes.

2. Materials and Methods

2.1. Experimental procedure

In this study, male Wistar rats (procured from Pasteur's institute), weighing 220-270 g were housed in an air-conditioned colony room on a light/dark cycle (21 ± 2°C and 30-40% humidity) and supplied with standard diet and tap water. Procedures involving animals and their care were conducted in conformity with the institutional guidelines and in accordance with the NIH guidelines for the care and use of laboratory animals.

The animals (n = 20) were randomly divided into two experimental groups: control and diabetic. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/Kg) dissolved in cold 0.9% saline solution immediately before use. Diabetes was verified by a serum glucose level higher than 250 mg/dl using glucose oxidation method (glucose oxidase kit, Zistchimie, Tehran).

2.2. Preparation of aortic rings

After 4 weeks, the rats were anesthetized with diethyl ether, decapitated, and through opening the abdomen, descending thoracic aorta was carefully excised and placed in cold physiological saline solution (PSS) containing (mM): NaCl 118, KCl 4.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11.1, $NaHCO_3$ 27.2, ethylenediaminetetraacetic acid (EDTA) 0.03, and $CaCl_2$ 1.8. Thereafter, the aorta was cleaned of excess connective tissue and fat and cut into rings of approximately 4 mm in length. Aortic rings were suspended between two triangular-shaped wires. One wire was attached to a fixed tissue support in a 50 ml isolated tissue bath containing PSS (pH 7.4) maintained at 37°C and continuously aerated with a mixture of 5% CO_2 and 95% O_2. The other end of each wire attached by a cotton thread to a isometric force transducer (UgoBasile, Italy) coupled to a signal amplifier and connected to a computer via an A/D interface. Recording and analysis of data was performed using a customized software. In all experiments, special care was taken to avoid damaging the luminal surface of endothelium. The rings were allowed to equilibrate for 90 min under a resting tension of 1.5 g before experiments were begun. This had

been shown in preliminary experiments to be the optimal resting tension for all groups. During equilibration period, the rings were washed every 30 min. For examining the endothelial integrity, pre-constricted rings with phenylephrine (PE, 1 μM) were exposed to a single addition of acetylcholine (ACh, 10 μM).

After an initial equilibration, the aortic rings were allowed to achieve maximal tension by exposure to high K^+ solution (80 mM), which was prepared by replacing the NaCl concentration of PSS with an equimolar concentration of KCl. Then, the relaxant responses to different concentrations of extract (0.2-1 mg/ml) were recorded. After PSS rinsing (3 times within a period of 30 min), the rings were constricted with PE (1μM) and again the relaxant responses to the same concentrations of the extract were recorded. The extract-evoked vasorelaxation was expressed as a percentage of relaxation and the IC_{50} (the concentration which produced a 50% maximal relaxation) was determined from the concentration-response curves.

The involved mechanisms underlying the vasorelaxant action of the extract was examined by pretreatment of the aortic rings with nitro-L-arginine-methyl ester (L-NAME, 100 μM) and indomethacin (10 μM) individually or in combination 30 min before addition of the vasoconstrictors and the extract.

In order to determine the involvement of intracellular Ca^{+2} mobilization in the vasorelaxant action of the extract, a Ca^{+2}-free PSS prepared by replacing CaCl2 in PSS with an equimolar concentration of $MgCl_2$ and the addition of ethylene-glycol-tetraacetic acid (EGTA, 0.5 mM) to chelate any free Ca^{+2} in the medium. After a preincubation period in this solution for 15 min with 3-4 serial washings, PE (1 μM) was added to stimulate the release of intracellular Ca^{+2} and the contraction recorded for at least 2 min. A similar procedure was repeated with Ca^{+2}-free PSS containing the extract (1 mg/ml).

2.3. Drugs and chemicals

Phenylephrine, acetylcholine-HCl, indomethacin, L-NAME and streptozotocin were purchased from Sigma Chemical (St. Louis, Mo., USA). All other chemicals were purchased from Merck (Germany) and local market. Indomethacin was dissolved in 0.5% w/v sodium bicarbonate. Further dilutions of the drugs were made in PSS. Meanwhile, streptozotocin was freshly dissolved in 0.9% saline solution.

2.4. Extraction

MO aerial part was dried under shade at room temperature. Thereafter, 100 g of its powder was mixed with 1000 ml of distilled boiling water for a period of 10 min under continuous stirring. The obtained mixture was filtered twice through a mesh and then one time through a filtered funnel, and the obtained liquid was dried on a magnet stirrer until a concentrated residue was obtained. This stock extract was maintained at −20 ◦C until being used. Lower concentrations of the extract were prepared by its dilution.

2.5. Data and statistical analysis

All values were given as means ± S.E.M. and were analyzed by student's t-test or one-way analysis of variance (ANOVA) followed by Tukey post-hoc test with a significant level of $P<0.05$.

3. Results

After 4 weeks, body weight of diabetic animals significantly decreased ($p<0.05$). Regarding serum glucose level, it significantly increased to 398.2 ± 19.6 mg/dl from the initial level of 131.8 ± 6.4 mg/dl ($p<0.0005$).

With respect to contractile response of aortic rings, PE (1 mM) induced a sustained contraction of the rat aorta with a peak tension of 514.6 ± 25.1 and 718.2 ± 13.5 mg in control and diabetic groups, respectively. This difference was not statistically significant ($p<0.01$). Addition of the extract to aortic rings from control and diabetic rats induced a dose-dependent relaxation of the pre-contracted rings with PE (Fig 1A). In this respect, extract-induced vasorelaxation of rings from diabetic group was significantly lower only at concentrations higher than 0.4 mg/ml as compared to control group ($p<0.05-<0.01$).

Addition of high K^+ (80 mM)-containing PSS to the tissue bath induced a maximal tension of
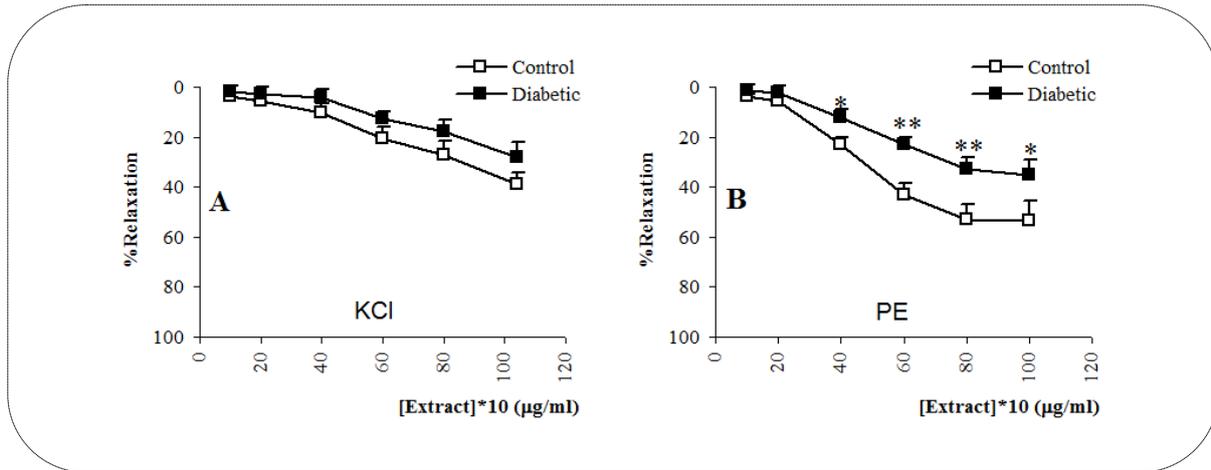
Figure 1. Vasorelaxant effect of MO extract against KCl (A) and PE (B)-induced contractions in aortic rings from control (n = 7) and streptozotocin-diabetic (n = 8) rats.
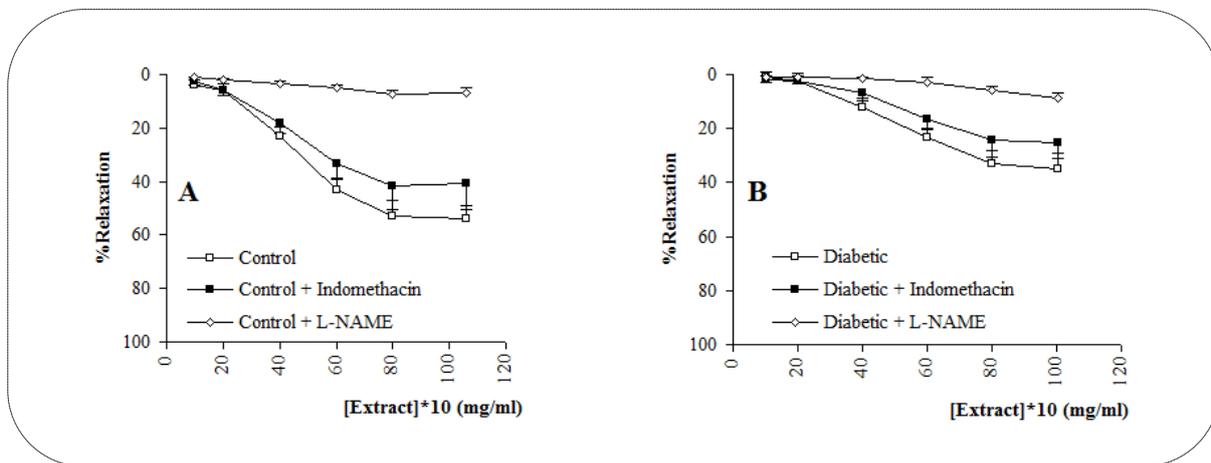* P<0.05, ** p<0.01 (versus diabetic)

Figure 2. Vasorelaxant effect of MO extract against PE-induced contractions in the presence of L-NAME or indomethacin in control (n = 8) (A) and diabetic (n = 7) (B) groups.
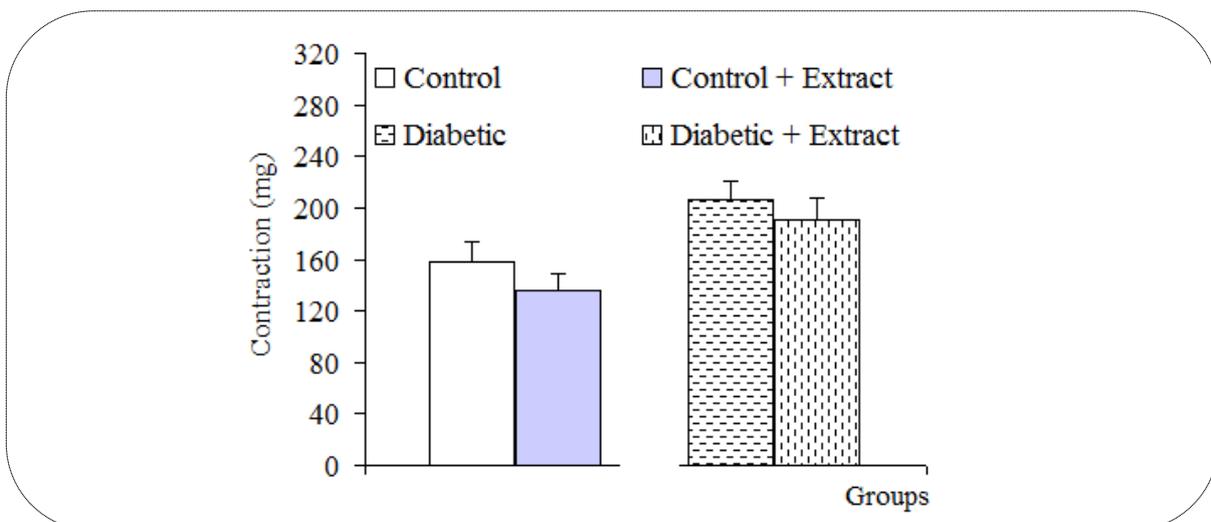

Figure 2. Effect of MO extract on PE-induced transient contractions in normal and Ca+2-free PSS in control (n = 6) and diabetic (n = 7) groups.

271.4 ± 14.8 and 293.4 ± 19.5 mg in control and diabetic groups respectively. The addition of extract produced a concentration-dependent relaxation in both control and diabetic groups (Fig. 1B) and the difference was not significant.

To further evaluate the mechanism of the vasorelaxant response in control and diabetic groups, the aortic rings were pre-incubated for 30 min with L-NAME (100 μM), a nitric oxide synthesis inhibitor, or indomethacin (10 μM) a cyclooxygenase inhibitor. Pretreatment of the tissues with L-NAME and not indomethacin markedly attenuated (but not abolished) the inhibitory effect of the extract against PE(1 μM)-induced contraction in both control (Fig. 2A) and diabetic groups (Fig. 2B).

In another series of experiments, the effect of extract was studied against contractions induced by PE in Ca^{+2}-free PSS. In the absence of extracellular Ca^{+2}, PE produced a transient contraction in control and diabetic groups. This difference was not significant statistically. Furthermore, pretreatment of the aortic rings with the extract did not significantly reduce the contractions induced by PE for both groups (Fig. 3).

4. Discussion

The present work was performed to investigate the underlying mechanisms involved in MO extract-induced vasorelaxation in PE- and KCl-precontracted aortae of subchronic STZ-diabetic rats. It was found out that MO induced a NO- and not prostraglandin-dependent vasorelaxation in aortic rings from diabetic rats.

There are two mechanisms to have the vasodilation response: the secretion of relaxant factor from vascular endothelium and inhibition of vasoconstriction. The former is mediated by bradykinin, prostacycline and NO (8). The relaxant action of MO extract was only affected by L-NAME, suggesting that the effect was mediated via endothelium-derived NO. NO which is produced by eNOS is a potent vasodilator by stimulating soluble guanylate cyclase and increasing cGMP levels in smooth muscle cells. Our results showed that pretreatment of aortic specimens with a nitric oxide synthase inhibitor, L-NAME significantly reduced the vasorelaxant effects of MO extract.

Indeed, MO extract concentration-dependently increased nitrite concentrations as well as eNOS activity in the rat aorta. Therefore, our findings may suggest that MO extract could relax the isolated rat aorta through endothelium-dependent NO pathway.

Vasodilator effects of flavonoids derived from medicinal plants have already been reported in the literature and most of the flavonoids described to date exhibit a relaxant effect independent of the presence of a functional endothelium (9). Previous reports have also shown vasodilator effect of MO extract in isolated rat aorta from normal rats (7). Although MO as a medicinal plant has been recorded for its utilization against some cardiac disorders, there is a restricted knowledge about its cardiovascular activity (7). Many earlier studies with plant extracts which have used similar experimental models proved that phenolic constituents such as flavonoids, coumarines, phenylethanoids, and other polyphenols are possibly accountable for vasorelaxant activity (10, 11). It is possible that some part of MO extract in our study could be attributed to such compounds.

These findings show that MO aqueous extract could relax the PE-preconstricted rings of aorta in STZ-diabetic rats through a nitric oxide pathway and prostaglandin pathway does not have a significant role and this extract could not affect the release and mobilization of calcium from intracellular stores.

References

1. Pedicino D, Liuzzo G, Trotta F, Giglio AF, Giubilato S, Martini F, et al. Adaptive immunity, inflammation, and cardiovascular complications in type 1 and type 2 diabetes mellitus. Journal of Diabetes Research 2013;2013:184258.

2. Patel VB, Parajuli N, Oudit GY. Role of angiotensin-converting enzyme 2 (ACE2) in diabetic cardiovascular complications. Clinical Science (London, England). 2014;126(7):471-82.

3. Lin K, Lloyd-Jones DM, Molitch ME, Li D, Carr JC. Quantitative imaging biomarkers for the evaluation of cardiovascular complications in type 2 diabetes mellitus. Journal of Diabetes and Its Complications 2013.

4. Kazemi S, Asgary S, Moshtaghian J, Rafieian M, Adelnia A, Shamsi F. Liver-protective effects of hydroalcoholic extract of allium hirtifolium boiss. In rats with alloxan-induced diabetes mellitus. ARYA Atherosclerosis 2010;6(1):11-5.

5. de Carvalho NC, Corrêa-Angeloni MJ, Leffa DD, Moreira J, Nicolau V, de Aguiar Amaral P, et al. Evaluation of the genotoxic and antigenotoxic potential of Melissa officinalis in mice. Genetics and Molecular Biology 2011;34(2):290-7.

6. Jun HJ, Lee JH, Jia Y, Hoang MH, Byun H, Kim KH, et al. Melissa officinalis essential oil reduces plasma triglycerides in human apolipoprotein E2 transgenic mice by inhibiting sterol regulatory element-binding protein-1c-dependent fatty acid synthesis. The Journal of Nutrition 2012;142(3):432-40.

7. Ersoy S, Orhan I, Turan NN, Sahan G, Ark M, Tosun F. Endothelium-dependent induction of vasorelaxation by Melissa officinalis L. ssp. officinalis in rat isolated thoracic aorta. Phytomedicine 2008;15(12):1087-92.

8. Mahdavi MR, Roghani M, Baluchnejadmojarad T. Mechanisms responsible for the vascular effect of aqueous Trigonella foenum-graecum leaf extract in diabetic rats. Indian Journal of Pharmacology 2008;40(2):59-63.

9. Abeywardena M, Runnie I, Nizar M, Suhaila M, Head R. Polyphenol-enriched extract of oil palm fronds (Elaeis guineensis) promotes vascular relaxation via endothelium-dependent mechanisms. Asia Pacific Journal of Clinical Nutrition. 2002;11 Suppl 7:S467-72.

10. Baluchnejadmojarad T, Roghani M, Jalali Nadoushan MR, Vaez Mahdavi MR, Kalalian-Moghaddam H, Roghani-Dehkordi F, et al. The sesame lignan sesamin attenuates vascular dysfunction in strepto-zotocin diabetic rats: involvement of nitric oxide and oxidative stress. European Journal of Pharmacology 2013;698(1-3):316-21.

11. Roghani M, Vaez Mahdavi MR, Jalali-Nadoushan MR, Baluchnejadmojarad T, Naderi G, Roghani-Dehkordi F, et al. Chronic administration of daidzein, a soybean isoflavone, improves endothelial dysfunction and attenuates oxidative stress in streptozotocin-induced diabetic rats. Phytotherapy Research 2013;27(1):112-7.