ORIGINAL ARTICLE

Vasorelaxant Activity and Its Mechanisms of *Momordica charantia* Fruit Extract in Diabetic Rat Aorta

Syafinaz Zainol[1], *Aidiahmad Dewa[2], Nornisah Mohamed[1], Nadiah Razali[2], Nurul Maizan Manshor[2], Rusdiah Ruzanna Jusoh[2,3]

[1] Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia
[2] Department of Physiology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia
[3] Manipal University College Malaysia, Jalan Batu Hampar, Bukit Baru, 75150 Melaka, Malaysia

ABSTRACT

Introduction: World Health Organisation estimated that diabetes mellitus (DM) was responsible for 1.6 million (4%) of 41 million deaths due to noncommunicable diseases globally in 2016. National Health and Morbidity Survey (2015) estimated that 3.5 million (17.5%) adult Malaysians were diabetic. Diabetic condition reduces the vascular endothelial functions, risking the individuals of developing hypertension. *Momordica charantia* is important in traditional folk medicine for managing hypertension and DM, however, knowledge on its vasorelaxant effect are still indifinite. This study investigated vasorelaxant activity and its mechanism of *M. charantia* fruit extracts in nondiabetic and diabetic Sprague Dawley rat aortic rings. **Methods:** *M. charantia* extracts and metformin were evaluated for vasorelaxant activity using endothelium-intact and endothelium-denuded nondiabetic and STZ-induced diabetic rat aortic rings. *M. charantia* Perlis acetone (MCRAC) extract was selected for the mechanism study. For vasoconstriction study, the aortic rings were challenged with phenylephrine. In vasorelaxation study, roles of nitric oxide, muscarinic cholinergic receptors, cGMP and cyclooxygenase pathways, K^+ and Ca^{2+} ions were investigated. **Results:** *M. charantia* extracts exhibited vasorelaxant capacity in both nondiabetic and diabetic conditions. Blood pressure lowering activity of MCRAC involved endothelium-derived NO and prostacyclin, cGMP pathway, muscarinic receptors, and potassium channel. Metformin reduced the blood pressure through endothelium-derived NO and prostacyclin, cGMP pathway, and muscarinic receptors. Diabetic condition also potentiated reduced extracellular and intracellular Ca^{2+} mobilities for both metformin- and MCRAC-mediated relaxation responses which were absent in nondiabetic condition. **Conclusion:** *M. charantia* extracts exerted blood pressure lowering activity in diabetic and nondiabetic states involving peripheral vascular pathways.

Keywords: *Momordica charantia* extract; Metformin; Vasorelaxation effect; Mechanism of action; Diabetic aortic ring

Corresponding Author:
Aidiahmad Dewa, PhD
Email: aidiahmad@usm.my
Tel: +604-6534589

INTRODUCTION

Diabetes mellitus is a great threat to public health. In 2016, the World Health Organisation (WHO) estimated that diabetes was responsible for 1.6 million (4%) out of 41 million deaths due to noncommunicable diseases globally (1). Based on the National Health and Morbidity Survey (NHMS) in 2015, it was estimated that 3.5 million (17.5%) adult Malaysians living with diabetes (2). Due to its long-term complication, diabetes can negatively affect various organs. Risk factors in diabetes involves dyslipidaemia, insulin resistance and atherosclerosis-induced oxidative stress, which could reduce the vascular endothelial functions. Sun et al. (2022) (3) reported that endothelial dysfunction has been considered as an important factor as well as the primary pathological change in the occurrence of diabetic vascular disease. The vascular endothelium plays an important role in controlling the balance factor that sustains the normal arterial function and vascular homeostasis. Functional impairment of endothelium-dependent vasodilation has been exhibited in numerous of different diabetic models. Vasodilation is one of the crucial physiological phenomena due to relaxation in smooth muscle cell, especially in the small arterioles, large arteries, and large veins. Vasodilation reduces high blood pressure and the increase of peripheral resistance produced by vasoconstriction, bringing to a decrease of the cardiac load (4).

Herbal medicines have been employed in the traditional approach of medicine for decades in various countries in the world. In accordance with the WHO guideline, ethno-medicinal plant investigations obtain more attention (5, 6). Numerous previous studies have

demonstrated the vasorelaxant properties of natural product that help to decrease blood pressure (7, 8). Bitter gourd or bitter melon, which is a climbing shrub, cultivated mainly in India, China, Bangladesh and Japan, commonly in Asian countries. Its scientific name is *M. charantia* and belongs to genus of the family Cucurbitaceae. Over the past decades, there is a large volume of published studies investigating the health and pharmacological activities of bitter melon. Clouatre et al. (2011) (9) revealed that bitter melon extract decreased systolic blood pressure in STZ-induced diabetic rats. Komolafe et al. (2014) (10) who studied effects of *M. charantia* on serum lipid profile of cardiovascular damage in diabetic rats, reported a significant reduce in the NO concentration in diabetic Wistar rats. Four weeks administration of *M. charantia* methanolic extract demonstrated a better protective potential on vascular system when compared to glimepiride. Based on the basis of all the findings, the purpose of the present work was to evaluate the underlying mechanisms of the vasorelaxant activity of different extracts of *M. charantia* that is still lacking.

MATERIALS AND METHODS

Drugs and Chemicals

Acetone were acquired from Merck Sdn Bhd (Selangor, Malaysia). Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), methylene blue (MB), indomethacin (INDO), atropine, glibenclamide, acetylcholine (ACh), phenylephrine (PE), sodium nitroprusside (SNP), potassium chloride (KCl), calcium chloride ($CaCl_2$) were purchased from Sigma-Aldrich Company (St Louis, Mo, USA). All drugs and chemicals used were analytical grade.

Plant material and crude extraction

M. charantia fruits, which were from Perlis, Johor, Pahang, Pulau Pinang, Selangor were obtained from Herbagus Sdn Bhd, Pulau Pinang, Malaysia. The plant was authenticated by Dr. Rahmad Zakaria (Herbarium of School of Biological Sciences, Universiti Sains Malaysia). A voucher specimen (No. 11727) was deposited at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The dried fruits were pulverised into a fine powder using an electric grinder (Retsch, Germany).

20 g powder of dried fruit of *M. charantia* was extracted with 200 mL of different solvent (water, water:ethanol (1:1), ethanol and acetone) using Soxhlet apparatus for 6 hours. The extract solution was evaporated to dryness using rotary evaporator R100 (Buchi, Switzerland) at 40°C. Then, the extracts were put in the oven at 45°C for 12 hour to ensure complete dryness.

Experimental animals

Adult male Sprague Dawley (SD) rats weighing 270 – 350 g were obtained from Animal Research and Service Centers (ARASC), Universiti Sains Malaysia and were kept in the animal transit room of School of Pharmaceutical Sciences, Universiti Sains Malaysia. The rats were acclimatised to laboratory conditions for 7 days (room temperature: 25 °C – 30 °C). The rats were maintained under constant environmental conditions with free access to commercial rodent pellet diet and water *ad libitium*. All procedures involving the rats were approved by the Animal Ethics Committee, Universiti Sains Malaysia, with protocol number USM/IACUC/2017/(107)(86). The rats were divided into three experimental groups: control (C), diabetic (D) and metformin (M) groups (6 rats in each group). Diabetes was induced by a single intraperitoneal injection of streptozotocin (diabetic, STZ, 60 mg/kg) after an overnight fast. Measurement of glucose levels in tail blood were performed using ACCU-CHEK Advantage-II Glucometer (Roche Diagnostics, Germany). Diabetes was confirmed by a consistent hyperglycaemia (blood glucose levels exceeded 11.0 mmol/L) after 3 days of injection (11). The rats were lefts with proper care for three weeks to develop vascular complications due to diabetic condition induced.

Preparation of aortic rings

The isolated thoracic aortic rings from diabetic and nondiabetic rats were prepared based on method by Ameer et al. (2009) (12) for the evaluation of vascular reactivity. The rats were anaesthetised using sodium pentobarbital (60 mg/kg, i.p). An incision through the sternum was performed to open up the thoracic cavity. The thoracic aorta was then located and carefully cleaned of fat and connective tissues. The aorta was subsequently immersed in a Krebs-Ringer bicarbonate solution composed of 118.6 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.2 mM KH_2PO_4, 25.1 mM $NaHCO_3$ and 11.0 mM glucose. The cleaned aorta was cut into 3-5 mm long rings and suspended horizontally on the tissue chamber containing 10 mL Krebs-Ringer bicarbonate solution. During the experiment, the Krebs solution was bubbled continuously with carbogen (95 % O_2 and 5 % CO_2) at 37 °C to prevent anoxic condition. The aortic rings were equilibrated under a tension of 1 g for 45 minutes. Every 30 minutes, the Krebs solution was replaced during equilibration. Contractions of aortic ring were induced by PE (1 μM) then followed by addition of acetylcholine (ACh, 1 μM) to establish the presence of intact and functional endothelium. The tension was determined with a force-displacement transducer (Grass FT03T) coupled with a data acquisition system (PowerLab; ADInstruments Pty Ltd, Australia). Data were analysed and stored using

LabChart 8 software (AD Instrument, Sydney, Australia). All the procedures were applied on both groups of rats; healthy groups (control) and diabetic groups.

Role of *M. charantia* acetone extracts on PE-induced contraction in diabetic aortic rings

The diabetic aortic rings were prepared as described in order to avoid any damage to the endothelium. Endothelium-intact rings were precontracted with 1 µM of PE. Once a constant plateau observed, 100 µL of *M. charantia* extracts (Perlis, Pulau Pinang, Pahang, Johor, Selangor) were cumulatively added (0.25 – 4.0 mg/mL) to the aortic rings. Each type of extract was evaluated in 6 diabetic aortic rings at each investigated concentration. Concentration-response relaxation following cumulative additions of *M. charantia* extracts were recorded using a force-displacement transducer (Grass FT03T) coupled with a data acquisition system (PowerLab; ADInstruments Pty Ltd, Australia). Data were analysed and stored using LabChart 8 software (AD Instrument, Sydney, Australia).

Role of MCRAC on PE-induced contraction in nondiabetic and diabetic aortic rings

A concentration-response curve was constructed by cumulative additions of PE (ranging from 10^{-9} to 10^{-5} M). The vasoconstriction effects of PE were investigated in endothelium-intact aortic rings using 3 different concentrations of *M. charantia* acetone extract from Perlis (MCRAC) (0.5, 1.0 and 2.0 mg/mL)(13).

Role of MCRAC on ACh- and SNP-induced relaxation in nondiabetic and diabetic aortic rings

Effects of MCRAC on the aortic ring responses to endothelium-intact and endothelium-denuded vasorelaxation were investigated through application of ACh or SNP, respectively. The aortic rings were precontracted with PE (1 µM) and followed by ACh or SNP (10^{-10} to 10^{-3} mol/L) being added cumulatively in nondiabetic and diabetic aortic rings. Effects of the vasodilators were determined for each concentration before and following tissue pretreatment with 2 mg/mL MCRAC for 30 minutes (14).

Role of L-NAME, indomethacin, atropine, methylene blue and glibenclamide on MCRAC-induced relaxation in nondiabetic and diabetic aortic rings

Assessment of vascular relaxation response of *M. charantia* on aortic rings was performed using endothelium-intact and -denude aortic rings with the presence and absence of different antagonist and agonist. The aortic rings were precontracted with PE (1 µM) and the vasorelaxant effects of cumulative concentration response of MCRAC (0.0001-3 mg/mL) were recorded for each concentration before and after aortic rings incubation with L-NAME (10 µM), indomethacin (10 µM), methylene blue (10 µM), atropine (1 µM) and glibenclamide (10 µM) for 15-20 minutes (14).

Role of MCRAC on extracellular Ca^{2+} influx and intracellular Ca^{2+} release in nondiabetic and diabetic aortic rings

Based on the method described by Senejoux et al. (2013) (15), two sets of experiments were carried out in endothelium-denuded aortic rings. These experiments evaluated the effect of MCRAC on extracellular Ca^{2+} uptake or intracellular Ca^{2+} release for the endothelium-denuded relaxation. In the initial stage, the denuded aortic rings were stabilised in normal Krebs - omit ‘ solution. Then, the normal Krebs - omit ‘ solution was replaced with Ca^{2+}-free solution containing 50 µM EGTA (ethylene glycol-bis (2-amino-ethylether)-N’,N’,N’,N’-tetra-acetic acid) for 15 minutes in order to eliminate Ca^{2+} from the tissue. The Ca^{2+}-free solution was replaced twice followed by addition of PE (1 µM) to produce steady contractions. Following 10 minutes incubation with MCRAC (2 mg/mL), Ca^{2+} was added cumulatively at concentrations of 0.01 to 3.0 mg/mL to obtain dose-response contraction curves.

In the second experiment, endothelium-denuded aortic rings were incubated in Ca^{2+}-free Krebs solution (consisting of EGTA) to study the function of mobilising Ca^{2+} from intracellular stores on MCRAC contraction inhibition. PE (1 µM) was added afterward to produce a steady-state transient contraction (T1). Krebs solution, consisted of EGTA and was Ca^{2+}-free, was then used to wash the aortic rings three times. Following stabilisation, the aortic rings were incubated for 15 to 20 minutes with 2 mg/mL of MCRAC before PE (1 µM) was added once more to capture a second transient contraction (T2). The ratio of the second transient contraction to the first was calculated as reported by Dimo et al. (2007) (16) and Senejoux et al. (2013) (15).

$$\text{Ratio of two concentrations} = \frac{\text{Second transient contraction (T2)}}{\text{First transient contraction (T1)}}$$

Role of metformin on diabetic aortic ring contraction

A concentration-response curve was constructed by additions of PE (ranging from 10^{-9} to 10^{-5} M) cumulatively. Contraction effects of PE were investigated in endothelium-intact diabetic aortic rings by using 3 different concentrations of metformin (0.1, 1.0 and 10 mM) (12).

Role of metformin on ACh- and SNP-induced relaxation in diabetic aortic rings

By the application of ACh and SNP, respectively, the effects of metformin on the aortic ring responses to endothelium-intact and endothelium-denuded vasorelaxation were explored. In diabetic aortic rings, additions of ACh and SNP (10^{-10} to 10^{-3} mol/L) cumulatively were performed after the aortic rings were precontracted with PE (1 µM). The effects of the vasodilators at each concentration were assessed before and after tissue pretreatment with 10 mM

metformin for 30 minutes (17).

Role of L-NAME, indomethacin, atropine and methylene blue on metformin-induced relaxation in diabetic aortic rings

Assessment of vascular relaxation response of metformin on aortic rings was performed on endothelium-intact and -denude with the presence and absence of different antagonists and agonists. The aortic rings were precontracted with PE (1 μM) and the vasorelaxant effects of cumulative concentration response of metformin (0.00001–30 mM) in aortic rings were recorded for each concentration before and after aortic rings incubation with L-NAME (10 μM), indomethacin (10 μM), methylene blue (10 μM), atropine (1 μM) and glibenclamide for 15-20 minutes (17).

Role of metformin on extracellular Ca^{2+} influx and intracellular Ca^{2+} releases in diabetic aortic rings

Based on the method described by Senejoux et al. (2013) (15), two sets of studies were carried out in diabetic aortic rings. These investigations were carried out to determine whether metformin-induced relaxation of the endothelium involved intracellular Ca^{2+} release or extracellular Ca^{2+} absorption. In the initial experiment, the normal Krebs - omit ' solution was used to stabilise the denuded aortic rings. To remove Ca^{2+} from the tissue, the regular Krebs - omit ' solution was then substituted with a Ca^{2+}-free solution containing 50 M EGTA (ethylene glycol-bis (2-amino-ethylether)-N',N',N',N'-tetra-acetic acid) for 15 minutes in order to eliminate Ca^{2+} from the tissue. The Ca^{2+}-free solution was replaced twice followed by addition of PE (1 μM) to produce steady contraction. Following 10 minutes incubation with metformin (10 mM), Ca^{2+} was added cumulatively at concentrations of 0.01 to 3.0 mg/mL to obtain dose-response contraction curves.

In the second experiment, for role of mobilisation of Ca^{2+} from intracellular stores on metformin contraction suppression, endothelium-denuded aortic rings were incubated in Ca^{2+}-free Krebs solution (consisted of EGTA). Then, PE (1 μM) was instilled to produce a steady-state contraction (contraction 1). The aortic rings were washed three times with Ca^{2+}-free Krebs solution (consisted of EGTA). After stabilisation, the aortic rings were incubated with 10 mM of metformin for 15-20 minutes before PE (1 μM) was instilled again to record second contraction (contraction 2). The ratio of contraction 2 over contraction 1 was calculated as described in earlier section. (Dimo et al. (16) and Senejoux et al. (15)).

Statistical analysis

All data were expressed as mean ± S.E.M. of six experiments. The concentration-response curves were fitted and compared by analysis statistical evaluation employing paired t-test, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. The analysis was performed using Graphpad Prism 8.0. The significance was set at $p<0.05$.

RESULTS

Role of *M. charantia* acetone extracts on PE-induced contraction in diabetic aortic rings

The vasorelaxant effects of *M. charantia* acetone extracts were presented in Fig. 1A. Additions of all extracts cumulatively evoked significant concentration-dependent relaxation of diabetic aortic rings precontracted with PE in comparison to control. Based on its highest R_{max} value and vasorelaxant ability at

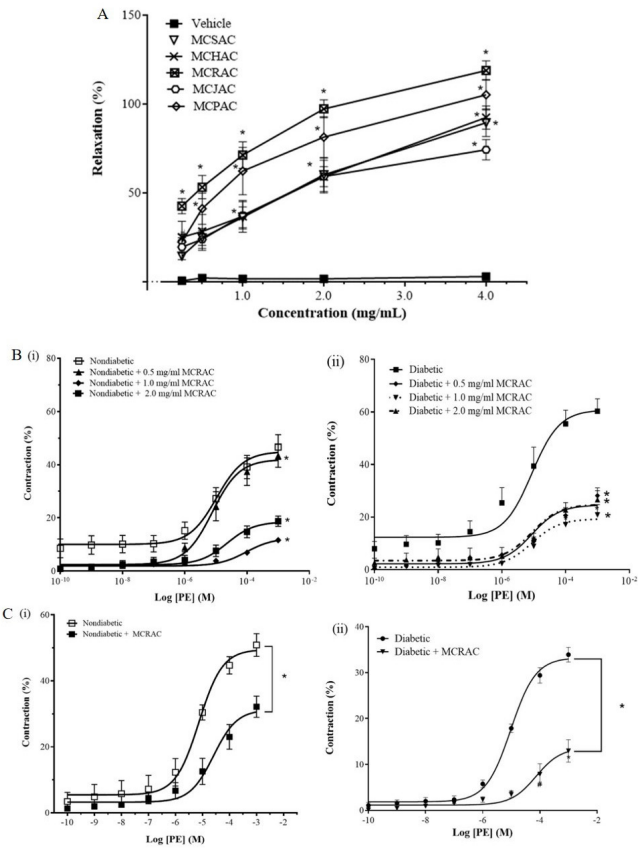

Figure 1 : A : Concentration-dependent relaxation induced by different *M. charantia* acetone extracts (Penang, MCPAC; Pahang, MCHAC; Perlis, MCRAC; Johor, MCJAC, Selangor, MCSAC, Control, normal saline) in endothelium-intact diabetic aortic rings contracted with PE (1 μM). Data were analysed using two-way ANOVA followed by Bonferroni post hoc test. Values are expressed as mean ± SEM (n = 6). * $p<0.05$ indicates significant difference as compared to control. **B :** cumulative addition of PE (10^{-10} to 10^{-3} M) in endothelium-intact aortic rings of (i) nondiabetic and (ii) diabetic groups. **C :** cumulative additions of PE (10^{-10} to 10^{-3} M) in endothelium-denuded aortic rings of (i) nondiabetic and (ii) diabetic groups. Data were presented as percentage contraction of concentration-response by PE. PE-induced contractions in the absence (control) and presence of MCRAC were analysed using one-way ANOVA and paired t-test.

the lowest concentration, MCRAC was thus selected for the mechanism of action study.

Role of MCRAC on PE-induced contraction in nondiabetic and diabetic aortic rings

Fig. 1B shows a significant ($p<0.05$) decrease in vasoconstrictions of the diabetic and nondiabetic aortic rings after incubation of the endothelium-intact aortic rings with different concentrations of MCRAC (0.5, 1.0, 2.0 mg/mL). A significant ($p<0.05$) drop in maximum response (C_{max}) was also observed in both diabetic and nondiabetic aortic rings. These results suggested that MCRAC decreased the vasoconstriction mediated by α1 adrenoceptor in both nondiabetic and diabetic conditions.

1.0 mg/mL MCRAC was selected for the mechanism study of the effects of MCRAC on the PE-induced contraction in endothelium-denuded aortic rings since there was no significant different between 1.0 mg/mL MCRAC and 2.0 mg/mL MCRAC.

PE-induced vasoconstrictions were significantly ($p<0.05$) reduced after preincubation of denuded aortic rings with 1.0 mg/mL MCRAC in nondiabetic and diabetic aortic rings, as compared to the control (Fig. 1C). This finding suggested that MCRAC reduced vascular contraction even in the absence of intact endothelium (endothelium independent) in both nondiabetic and diabetic conditions.

Role of MCRAC on ACh- and SNP-induced relaxation in nondiabetic and diabetic aortic rings

Additions of ACh (endothelium-intact vasodilator) (10^{-10} to 10^{-2} M) cumulatively to PE-precontracted aortic rings led to a concentration-dependent relaxation response in all groups. MCRAC significantly ($p<0.05$) increased ACh-induced relaxation in diabetic aortic rings but not in nondiabetic aortic rings as shown in Fig. 2A. These results suggested that MCRAC-mediated endothelium dependent vasorelaxation was potentiated in diabetes mellitus.

SNP (endothelium-denuded vasodilator), which was added cumulatively (10^{-10} to 10^{-3} M), produced relaxation on PE-preconstricted aortic rings in all groups. Pre-treatment with MCRAC significantly ($p<0.05$) increased the SNP-induced relaxation in a concentration-dependent manner both in nondiabetic and diabetic rat aortic rings (Fig. 2B). These results suggested that MCRAC produced endothelium-independent vasorelaxation, and this activity was not influenced by diabetes mellitus.

Role of L-NAME, indomethacin, atropine and methylene blue on MCRAC-induced relaxation in nondiabetic and diabetic aortic rings

MCRAC-induced vasorelaxation significantly ($p<0.05$) decreased after preincubation of nondiabetic and

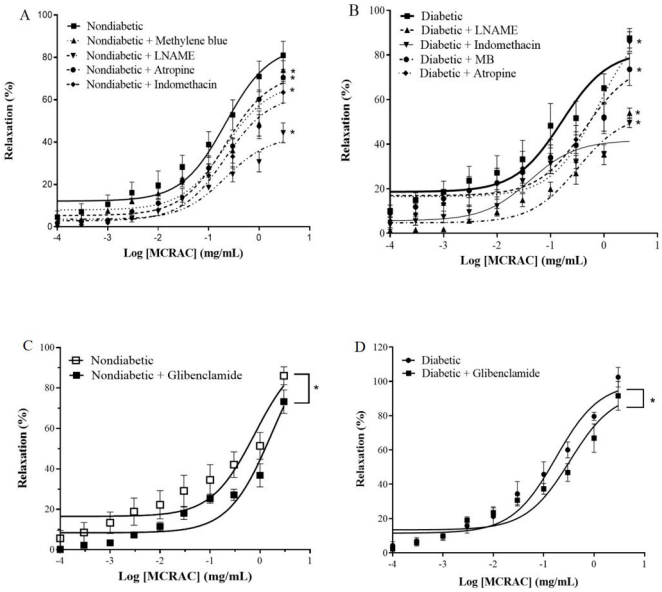

Figure 2 : Vasodilatory responses to MCRAC with 15-20 minutes preincubation of L-NAME (10 μM), atropine (1 μM), methylene blue (10 μM) and indomethacin (10 μM) treatment in **(A)** nondiabetic and **(B)** diabetic aortic rings. Vasodilatory responses to MCRAC with 15-20 minutes preincubation of glibenclamide (10 μM) treatment in **(C)** nondiabetic and **(D)** diabetic aortic rings. Data were analysed using paired t-test. Values are expressed as mean ± SEM of six (n=6) aortic ring experiments. * $p<0.05$ indicates significant difference compared before and after L-NAME, methylene blue, indomethacin, atropine and glibenclamide.

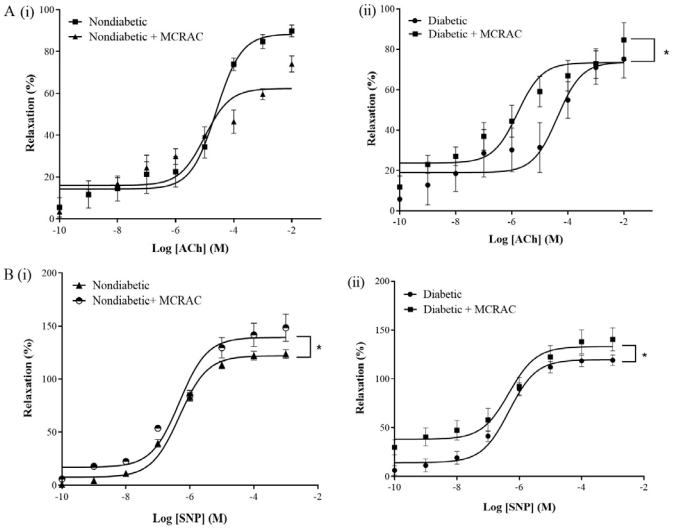

Figure 3 : Effects of MCRAC on acetylcholine-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from **A (i)** nondiabetic and **A (ii)** diabetic rats. Effects of MCRAC on sodium nitroprusside-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from **B (i)** nondiabetic and **B (ii)** diabetic rats. Data were analysed using paired t-test. Values are expressed as mean ± SEM of six (n=6) aortic ring experiments. * $p<0.05$ indicates significant difference compared before and after MCRAC.

diabetic aortic rings with L-NAME, indomethacin, atropine and methylene blue (Fig. 3A, 3B). These results suggested that endothelium-derived nitric oxide (EDNO), cyclooxygenase (COX) pathway, muscarinic receptors, and cGMP pathway played a role in MCRAC-mediated vascular relaxation both in nondiabetic and diabetic rat aortic rings, respectively.

Role of glibenclamide on MCRAC-induced relaxation in aortic rings

MCRAC-induced vasorelaxation were significantly ($p<0.05$) reduced after preincubation of nondiabetic and diabetic aortic rings with glibenclamide (Fig. 3C, 3D). These results proposed that potassium played a role in MCRAC-mediated vascular relaxation in both nondiabetic and diabetic aortic rings.

Role of MCRAC on extracellular Ca^{2+} influx and intracellular Ca^{2+} release in nondiabetic and diabetic aortic rings

Additions of $CaCl_2$ (0.01 to 3 mM) cumulatively on aortic rings induced gradual increased contraction on endothelium-denuded aortic rings (Fig. 6A). In the nondiabetic rings, MCRAC did not alter the Ca^{2+}-induced vasoconstriction. However, in the diabetic rings, extracellular Ca^{2+}-induced vasoconstriction was significantly ($p<0.05$) reduced by MCRAC. A similar observation was established in the evaluation of the intracellular Ca^{2+} release from the sarcoplasmic reticulum. MCRAC significantly ($p<0.05$) suppressed the attainment of maximal contraction induced by PE in diabetic rings but not in nondiabetic rings (Fig. 6B). These results suggested that diabetic mellitus probably enhance MCRAC's suppression of vasoconstrictions by reducing mobilisations of both extracellular and intracellular Ca^{2+} in diabetic aortic rings.

Role of metformin on diabetic aortic ring contraction

Pretreatment of the diabetic aortic rings with different concentrations of metformin (0.1, 1.0 and 10.0 mM) did not show any significant decrease in vasoconstriction of diabetic aortic rings (Fig. 5A). Additions of PE (10^{-10} to 10^{-2} M) cumulatively caused a concentration-dependent contraction response. Pretreatment of the aortic rings with 10 mM metformin led to a significant ($p<0.05$) reduction in vasoconstriction of the diabetic rings (Fig. 5B). This finding suggested that metformin's attenuation of PE-induced vasoconstriction in diabetic aortic rings was endothelium independent.

Role of metformin on ACh- and SNP-induced relaxation in diabetic aortic rings

Additions of ACh (endothelium-intact vasodilator) (10^{-10} to 10^{-2} M) cumulatively to PE-preconstricted aortic rings produced concentration-dependent relaxation responses. These relaxation responses were significantly ($p<0.05$) enhanced after incubation with metformin as compared to control as shown in Fig. 5C. This result suggested that metformin potentiated

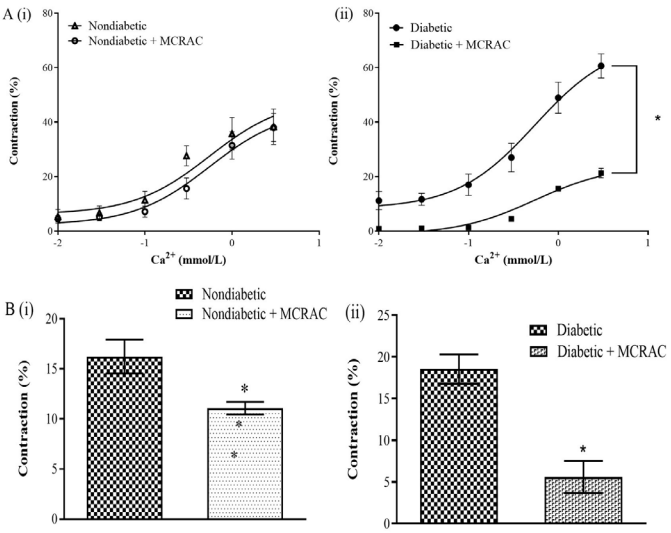

Figure 4 : A : Contribution of extracellular Ca^{2+} uptake in MCRAC-induced relaxation in the endothelium-denuded aortic ring of **(i)** nondiabetic and **(ii)** diabetic rats. **B :** Effects of MCRAC on phenylephrine-induced (PE,1 μM) contraction on endothelium denuded aortic rings in Ca^{2+} free Krebs solution with and without incubation of MCRAC (1 mg/mL) in **(i)** nondiabetic and **(ii)** diabetic aortic rings. Data were analysed using paired t-test. Values are expressed as mean ± SEM of six (n=6) aortic ring experiments. * $p<0.05$ indicates significant difference compared before and after MCRAC.

vascular relaxation mediated by muscarinic cholinergic receptors on diabetic aortic rings.

Additions of SNP (endothelium-denuded vasodilator) cumulatively (10^{-10} to 10^{-3} M) produced increased relaxation of PE-preconstricted aortic rings (Fig. 5D). Metformin significantly ($p<0.05$) enhanced relaxation responses as compared to control group. This result suggested that vascular relaxation of metformin in diabetic aortic rings was not dependent on the presence of intact endothelium.

Role of L-NAME, indomethacin, atropine and methylene blue on metformin-induced relaxation in diabetic aortic rings

Metformin-induced relaxation responses were significantly ($p<0.05$) reduced after preincubation of aortic rings with L-NAME, indomethacin, atropine, and methylene blue (Fig. 6). This finding suggested that metformin-mediated vascular relaxation in diabetic aortic rings involved endothelium-derived nitric oxide (EDNO) pathway, cyclooxygenase (COX) pathway, muscarinic receptors and cGMP pathway, respectively.

Role of glibenclamide on metformin-induced relaxation in diabetic aortic rings

Metformin-induced relaxation responses were not significantly ($p<0.05$) changed after preincubation of diabetic aortic rings with glibenclamide as compared to

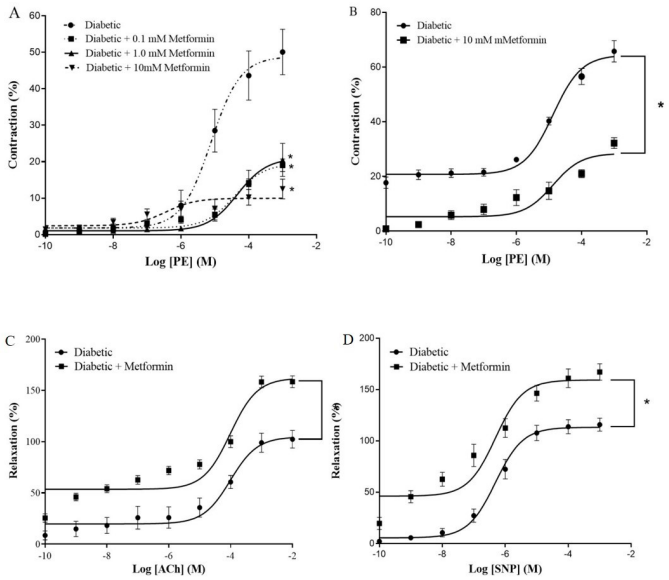

Figure 5 : Effects of different concentrations of metformin on the contractile responses induced by cumulative additions of PE (10^{-10} to 10^{-3} M) in **(A)** endothelium-intact and **(B)** endothelium-denuded aortic rings of diabetic rats. Data were presented as percentage contraction of concentration-response by PE. PE-induced contractions in the absence (control) and presence of metformin were analysed using paired t-test and two-way ANOVA followed by Bonferroni post hoc test. Values are expressed as the mean ± SEM of six (n=6) aortic ring experiments.* $p<0.05$ indicates significant difference compared to control. Effects of metformin on **(C)** acetylcholine-induced and **(D)** sodium nitroprusside-induced relaxation of phenylephrine-contracted endothelium-intact aortic rings from diabetic rats. Data were analysed using paired t-test. Values are expressed as mean ± SEM (n = 6 aortic rings). * $p<0.05$ indicates significant difference compared before and after metformin.

control (Fig. 10 A). This result suggested that potassium ionic movement played no role in metformin-mediated vascular relaxation.

Role of metformin on extracellular Ca^{2+} influx and intracellular Ca^{2+} release in diabetic aortic rings

Additions of $CaCl_2$ (0.01 to 3 mM) cumulatively to endothelium-denuded diabetic aortic rings in high K^+, Ca^{2+} free Krebs - omit ' solution produced gradual contractions. Preincubation of the diabetic aortic rings with metformin significantly ($p<0.05$) reduced the Ca^{2+}-mediated contraction as compared to control (Fig. 10B (i)). To evaluate role of intracellular Ca^{2+} release from the sarcoplasmic reticulum, preincubation with metformin significantly ($p<0.05$) suppressed the attainment of maximal contraction induced by PE as compared to the control (Fig. 10B (ii)). These results suggested that metformin-induced relaxation in diabetic aortic rings by suppressing mobilisation of both extracellular Ca^{2+} and intracellular Ca^{2+} in the vascular smooth muscles.

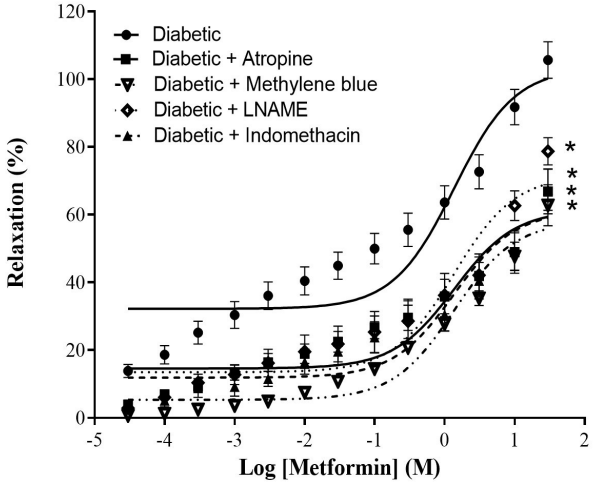

Figure 6 : Vasodilatory responses to metformin with 15-20 minutes preincubation of L-NAME (10 µM), atropine (1 µM), methylene blue (10 µM) and indomethacin (10 µM) treatment in diabetic aortic rings. Data were analysed using paired t-test. Values are expressed as mean ± SEM of six (n=6) aortic ring experiments. * $p<0.05$ indicates significant difference compared before and after L-NAME, methylene blue, indomethacin and atropine.

DISCUSSION

The present study was the first assessment to evaluate the mechanism of vasorelaxant activity of *M. charantia* fruit in diabetic and nondiabetic rat aortic rings. Loh et al. (18) performed a study on vasorelaxation activity of Uncaria rhynchophylla ethanolic extract using three different types of solvents and reported that different solvent exhibited different vasorelaxation activity. The present study also produced a similar pattern of results. Evaluation of the vasorelaxant activity of *M. charantia* different types of extracts (water, ethanol, water:ethanol (1:1), acetone) and different locations were performed using STZ-diabetic rat aortic rings. All extracts exhibited good vasorelaxant effects, however, MCRAC was selected for the mechanism study due to its ability to produce significant relaxation at the lowest concentration (0.25 mg/mL) when compared to the control and other extracts. It was postulated that vasoactive compounds extracted by acetone contributed to the highest vasorelaxant activity. A study performed by Lamai et al. (19) revealed that a flavonoid, morelloflavone isolated from Garcinia dulcis acetone extract exhibited vasorelaxant effect on isolated rat thoracic aortic rings. Calfio et al. (20) reported potent vasodilator activity of calafate berries extracted using mixture of acetone, water and acetic acid (50:49:1).

PE, an α1 adrenoreceptor agonist, induces aortic contraction by extracellular Ca^{2+} influx through receptor-operated calcium channels as well as by releasing of intracellular Ca^{2+} from sarcoplasmic reticulum (21).

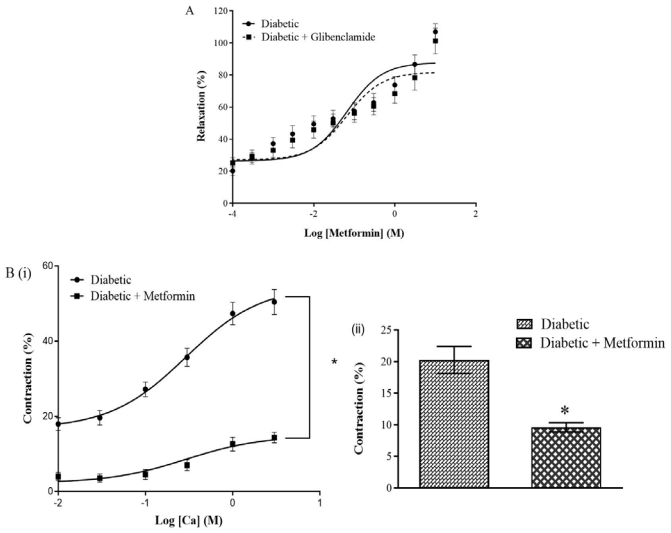

Figure 7 : A : Vasodilatory responses to metformin with 15-20 minutes preincubation of glibenclamide (10 μM) treatment in diabetic group. Data were analysed using paired t-test followed by Bonferroni post hoc test. Values are expressed as mean ± SEM (n = 6 aortic rings). * $p<0.05$ indicates significant difference compared before and after glibenclamide. **B:** Effects of metformin on extracellular and intracellular Ca^{2+} influx on metformin-induced relaxation in diabetic aortic rings. Data were analysed using paired t-test. Values are expressed as mean ± SEM of six (n=6) aortic ring experiments. * $p<0.05$ indicates significant difference compared before and after metformin.

The latter pathway relates to the PE stimulation of phospholipase C to generate diacylglycerol and 1,4,5 triphosphate inositol (IP_3). Consequently, diacylglycerol activates the light chain of myosin by protein kinase C activation and IP_3 receptors trigger Ca^{2+} release from the sarcoplasmic reticulum via opening of IP_3 receptors (20). Niazmand et al. (22) reported that relaxant activity of *N. sativa* seed on the contractions induced by PE in vascular smooth muscle cells were associated with inhibition of extracellular Ca^{2+} influx as well as suppression of IP_3-mediated receptors. The present study showed that MCRAC elicited concentration-dependent relaxation effects on PE-induced vasocontraction of diabetic aortic rings, and this may because of these effects. It was observed that the C_{max} value of diabetic aortic rings was higher than the nondiabetic aortic rings. The increased aortic contraction effects of diabetic rats may be associated to impairment of endothelial function (23), oxidative stress (24), enhanced Ca^{2+} influx via voltage dependent L-type calcium channels (25) and enhanced vasoconstrictor prostanoids due to rise of superoxide anions and enhanced sensitivity to adrenergic agonists (26). Besides, it was also found that MCRAC significantly reduced the vasoconstriction effects on endothelium-denude in diabetic and nondiabetic aortic rings. These findings postulated that the blood pressure lowering effects of MCRAC through vascular contraction reduction were both endothelium-dependent and endothelium-independent in nature.

In endothelial cells of majority vascular beds, ACh is capable to provoke formation and release of endothelial-derived relaxing factors such as prostacyclin, nitric oxide as well as endothelial-derived hyperpolarising factor. This pathway leads to vascular smooth muscle relaxation in an endothelium-intact manner (27). The ACh-induced relaxation response involves nitric oxide-mediated and endothelium-intact. In this study, MCRAC-induced ACh-relaxation of the aortic rings from both nondiabetic and diabetic rats. These results further suggested that MCRAC's blood pressure lowering effects were achieved through reduction of vascular contraction and/or enhancement of vascular relaxation.

Impairment of vasorelaxation responses could also be associated to changes in smooth muscle as proposed by the blunted response discovered in response to SNP. To establish whether endothelium was a critical factor for MCRAC to exhibit its vascular activity, SNP was applied to endothelium denude aortic rings in before and after preincubation with MCRAC. MCRAC potentiated SNP-induced relaxation in both diabetic and nondiabetic aortic rings suggested that its vascular relaxation was endothelium independent. This finding was congruent with that of Pinna et al. (28) that supplementation of vitamin C or grape seed proanthocyanidins preserved vascular response to SNP.

In general, two mechanisms contribute for the vasorelaxation response in the vascular system, namely through secretion of relaxant factors from the endothelium and inhibition of vasoconstriction by the vascular smooth muscle. The former involves prostacyclin, bradykinin and nitric oxide (29). To our knowledge, this was the first investigation that studied the mechanisms for the vasorelaxation effect of *M. charantia* extract in diabetic rat aortic rings.

It has become the topic of interest on the effects of chronic hyperglycaemia on production and release of nitric oxide from endothelial cells (30). In our study, L-NAME, a non-selective nitric oxide synthase inhibitor and methylene blue, a guanylate cyclase inhibitor, were employed to evaluate the participation of endothelium-derived NO and cGMP in the MCRAC-induced vasorelaxation. L-NAME and methylene blue significantly reduced the MCRAC-induced relaxation of the aortic rings in both nondiabetic and diabetic, suggesting the involvement of endothelium derived NO and cGMP pathways. This finding was in agreement with a study on zingerone, the main constituent of ginger by Ghareib et al. (31) which enhanced vascular contraction in diabetic aortic rings that could be contributed by vasorelaxation effects via NO- and

guanylate cyclase stimulation.

It is generally known that vasorelaxation of muscarinic receptors is mainly mediated through the production of NO in endothelial cells, which is activated by induction of Ca^{2+}-calmodulin complex and the stimulation of endothelial nitric oxide synthase (eNOS) (32). When challenged with atropine, a muscarinic receptor blocker, vascular relaxation by MCRAC exhibited significant reductions in both nondiabetic and diabetic, suggesting that muscarinic receptors were involved in the MCRAC vascular relaxation, and the diabetic condition did not cause any effect. Cechinel-Zanchett et al. (33) reported similar results as they investigated vascular effects of Bauhinia forficate leaves preparations in aortic rings of normotensive and hypertensive rats.

The present investigation also involved the application of indomethacin, a cyclooxygenase inhibitor. It was observed that indomethacin decreased MCRAC-induced relaxation suggesting the involvement of prostacyclin pathway. Endothelium-derived hyperpolarising factor stimulate endothelium-intact relaxation which is resistant to the combined inhibition of cyclooxygenase and NOS (34). Another suggested mechanism of EDHF-induced vasorelaxation include the arachidonic acid metabolism via the cytochrome P450 epoxynase pathway to the formation of epoxyeicosatrienoic acids (35).

Potassium channels are essential in the regulation of vascular tone and muscle contractility. The rise in K^+ permeability is contributed by membrane hyperpolarisation and thus lead to vasorelaxation (36). In the vasculature, NO stimulates potassium channels leading to smooth muscle relaxation (37). In the present study, to evaluate the role of potassium channels on the MCRAC-induced vasorelaxation, glibenclamide, an ATP sensitive potassium channels inhibitor was applied. Both aortic rings of nondiabetic and diabetic had their MCRAC-induced relaxation dropped suggesting the involvement of potassium channels in the MCRAC-induced relaxation.

Roles of Ca^{2+}-induced contraction in blood pressure lowering effects of MCRAC was investigated since Ca^{2+} is directly involved in the contraction of vascular smooth muscle. Both intracellular and extracellular Ca^{2+}-induced contractions were attenuated by MCRAC in diabetic aortic rings only. Diabetic mellitus probably enhance MCRAC's suppression of vasoconstrictions by reducing mobilisations of both extracellular and intracellular Ca^{2+} in diabetic aortic rings. The regulation of Ca^{2+} influx via cell membrane resulting from receptor-operated calcium channels (ROCC) and voltage dependent-type calcium channels (38, 39). Similar finding was reported by Lee et al. (40) as they investigated the vasorelaxant effect of *Sigesbeckia glabrescens* methanol extract on rat aortic rings and reported that the mechanism involved endothelium-denuded pathways which were associated to blockade of extracellular Ca^{2+} influx through receptor-operated calcium channels as well as voltage-dependent calcium channels.

Metformin, a biguanide, is one of the most commonly applied drugs to treat diabetes mellitus patients. Besides improving insulin sensitivity and reducing hyperglycaemia, it also independently contributes to vasculoprotection (41-43). Recently, it has been proposed that metformin enhances endothelial function in animal model and humans (44,45). Metformin restores the reactivity of microvascular to bradykinin, histamine or acetylcholine of venules of arteriols from neonatal SZ-diabetic rats (46). In our study, pretreatment of aortic ring with metformin significantly decreased contractions in endothelium-denuded diabetic aortic rings to PE at all concentrations. ACh and SNP-induced relaxations were examined in diabetic rat aortic rings with the presence of metformin. The results demonstrated that metformin pretreatment had significantly increased the vasorelaxant responses to ACh and SNP, suggesting that metformin-induced relaxation were mediated by endothelium-dependent and -independent factors/pathways. This was concurrent with the study by Azemi et al. (47) who reported that the increased PE-induced contraction as well as impaired acetylcholine-induced relaxation in diabetic rat aortic rings were restored after metformin treatment.

The present investigation also demonstrated that pretreatment with L-NAME, methylene blue and indomethacin significantly decreased metformin-induced relaxations in the endothelium-intact diabetic aortic rings but atropine did not. Thus, the results proposed that endothelium-derived NO, cGMP and cyclooxygenase pathway, but not muscarinic receptors, played important roles in metformin-mediated vascular relaxation. Similar findings were reported by Majithiya and Balaraman (48) that pretreatment with L-NAME blocked the increased ACh-induced relaxation in metformin-treated STZ-diabetic rats.

In this study, metformin-mediated relaxation was not influenced by potassium channels. This finding was in contrast with the study reported by Zhao et al. (2014) (49) that metformin restored the impaired of intermediate-conductance Ca^{2+}-activated potassium channel and small-conductance Ca^{2+}-activated potassium channel mediated vasorelaxation in rat mesenteric artery from STZ-induced diabetic rats. Metformin induced relaxation in diabetic aortic rings by suppressing mobilisation of both extracellular Ca^{2+} and intracellular Ca^{2+} in the vascular smooth muscles. Our results agreed with Dominguez et al. (50) that metformin attenuated thrombin-induced elevation in

cytosolic free Ca^{2+}.

CONCLUSION

It can be concluded that *M. charantia* extracts may exert a blood pressure lowering activity in diabetic and nondiabetic states involving peripheral vascular pathways. *M. charantia* extracts from five different states exhibited a potent vasorelaxant capacity in both nondiabetic and diabetic conditions. Blood pressure lowering activity of MCRAC, based on the aortic ring approach, was achieved through involvements of endothelium-derived relaxing factors (EDRFs) such as EDNO and prostacyclin, soluble guanylyl cyclase of cGMP pathway, muscarinic receptors, and potassium channels. Diabetic condition potentiated reduced mobility of extracellular and intracellular Ca^{2+} for MCRAC relaxation response which was not observed in nondiabetic condition. Metformin produced its blood pressure lowering activity through involvement of endothelium-derived EDNO and prostacyclin, soluble guanylyl cyclase of cGMP pathway, and muscarinic receptors. Diabetic condition also potentiated reduced mobility of extracellular and intracellular Ca^{2+} for metformin-mediated relaxation response which was not observed in nondiabetic condition.

ACKNOWLEDGEMENT

This work was supported by Mybrain15 scholarship under National Higher Education Strategic Plan from Ministry of Higher Education, Malaysia. The authors would like to express our gratitude to the School of Pharmaceutical Science, Universiti Sains Malaysia for the laboratory facility.

REFERENCES

1. Noncommunicable disease country profiles 2018. Geneva: World Health Organization; 2018 [cited 2020 Jun 7]. Available from https://apps.who.int/iris/handle/10665/274512.
2. Institute for Public Health and Morbidity Survey 2015. Kuala Lumpur; Ministry of Health Malaysia; 2015. Available from https://www.moh.gov.my/moh/resources/nhmsreport2015vol2.pdf.
3. Sun H, Saeedi P, Karuranga S, Pinkepank M, Ogurtsova K, Duncan BB, Stein C, Basit A, Chan JC, Mbanya JC, Pavkov ME. IDF Diabetes Atlas: Global, regional and country-level diabetes prevalence estimates for 2021 and projections for 2045. Diabetes Research And Clinical Practice. 2022; 183, 109119.
4. Chang TT., and Chen, J. W. Potential Impacts of Hydralazine as a Novel Antioxidant on Cardiovascular and Renal Disease—Beyond Vasodilation and Blood Pressure Lowering. Antioxidants. 2022; 11(11), 2224.
5. Benzi G, and Ceci A. Herbal medicines in European regulation. Pharmacological Research. 1997; 35 (5), 355–362.
6. World Health Organization. Guidelines for the Appropriate Use of Herbal Medicines Western Pacific Series, vol. 23. W.H.O. WHO regional publications, 1998; pp. 88.
7. Salahdeen HM, Idowu GO, Salami S., Murtala BA, Alada AA. Mechanism of vasorelaxation induced by Tridaxprocumbens extract in rat thoracic aorta. Journal of Intercultural Ethnopharmacoly. 2016; 5, 174–179.
8. Takashima M, Kanamori Y, Kodera Y, Morihara N Tamura K. Aged garlic extract exerts endothelium-dependent vasorelaxant effect on rat aorta by increasing nitric oxide production. Phytomedicine. 2017;24, 56–61.
9. Clouatre DL, Rao SN and Preuss HG. Bitter melon extracts in diabetic and normal rats favourably influence blood glucose and blood pressure regulation. Journal of Medicinal Food (2011) VOL. 14, NO. 12
10. Komolafe OA, Ofusori DA, Adewole OS and Fakunle JB. Effects of *Momordica charantia* on serum lipid profile, serum protein levels and selected markers of cardiovascular damage in diabetic rats. British Journal of Pharmaceutical Research. 2014; 4(4): 429-442, 2014.
11. Tang ST, Zhang Q, Tang HQ, Wang CJ, Su H, Zhou Q, et al..Effects of glucagon-like peptide-1 on advanced glycation endproduct-induced aortic endothelial dysfunction in streptozotocin-induced diabetic rats: Possible roles of Rho kinase-and AMP kinase-mediated nuclear factor κB signalling pathways. Endocrine. 2016; 53(1), 107-116.
12. Ameer OZ, Salman IM, Siddiqui MJ, Yam MF, Sriramaneni RN, Sadikun A, et al.. In vitro cholinomimetic effect of Loranthus ferrugineus in isolated guinea pig ileum. Journal of Acupuncture and Meridian Studies. 2009; 2(4):288-93.
13. Bello I, Usman NS, Mahmud R, Asmawi MZ. Mechanisms underlying the antihypertensive effect of Alstonia scholaris. Journal of Ethnopharmacology. 2015; 175, 422-431.
14. Razali N, Dewa A, Asmawi MZ, Mohamed N, Manshor NM. Mechanisms underlying the vascular relaxation activities of Zingiber officinale var. rubrum in thoracic aorta of spontaneously hypertensive rats. Journal of Integrative Medicine, 2020; 18(1), 46-58.
15. Senejoux F, Demougeot C, Cuciureanu M, Miron A, Cuciureanu R, Berthelot A, et al. Vasorelaxant effects and mechanisms of action of Heracleum sphondylium L. (Apiaceae) in rat thoracic aorta. Journal of Ethnopharmacology. 2013; 147, 536–539.
16. Dimo T, Bopda Mtopi OS, Nguelefack TB, Kamtchouing P, Zapfack L, Asongalem EA, et al. Vasorelaxant effects of Brillantaisia nitens Lindau (Acanthaceae) extracts on isolated rat vascular

smooth muscle. Journal of Ethnopharmacology. 2007; 111, 104–109.
17. Manshor NM, Razali N, Jusoh RR, Asmawi MZ, Mohamed N, Zainol S, et al.. Vasorelaxant effect of water fraction of Labisia pumila and its mechanisms in spontaneously hypertensive rats aortic ring preparation. 2020; International Journal of Cardiology Hypertension, 4, 100024.
18. Loh YC, Ch'ng YS, Tan CS, Ahmad M, Asmawi MZ, Yam MF. Mechanisms of action of Uncaria rhynchophylla ethanolic extract for its vasodilatory effects. Journal of Medicinal Food, 2017; 20(9), 895-911.
19. Lamai J, Mahabusarakam W, Ratithammatorn T, Hiranyachattada S. Effects of morelloflavone from Garcinia dulcis on vasorelaxation of isolated rat thoracic aorta. Journal of Physiological and Biomedical Sciences; 2013; 26(1), 13-17.
20. Calfío C, Huidobro-Toro JP. Potent vasodilator and cellular antioxidant activity of endemic patagonian calafate berries (Berberis microphylla) with nutraceutical potential. Molecules.2019; 24(15), 2700.
21. Thorneloe KS, and Nelson MT. Ion channels in smooth muscle: Regulators of intracellular calcium and contractility. Canadian Journal of Physiology and Pharmacology. 2005; 83(3), 215-242.
22. Niazmand S, Fereidouni E, Mahmoudabady M. Mousavi SM.. Endothelium-independent vasorelaxant effects of hydroalcoholic extract from Nigella sativa seed in rat aorta: The roles of Ca^{2+} and K+ channels. BioMed Research International, 2014.
23. Potenza MA, Gagliardi S, Nacci C, Carratu MR, Montagnani M. Endothelial dysfunction in diabetes: From mechanisms to therapeutic targets. Current Medicinal Chemistry, 2009;16(1), 94-112.
24. Tabit CE, Chung WB, Hamburg NM, and Vita JA. Endothelial dysfunction in diabetes mellitus: Molecular mechanisms and clinical implications. Reviews in Endocrine and Metabolic Disorders. 2010; 11(1), 61-74.
25. Pinho RAD, Araújo, MCD, Ghisi GLDM. Benetti, M. Coronary heart disease, physical exercise and oxidative stress. Arquivos Brasileiros de Cardiologia. 2010; 94, pp.549-555.
26. Ahmad S, Beg, ZH. Elucidation of mechanisms of actions of thymoquinone-enriched methanolic and volatile oil extracts from Nigella sativa against cardiovascular risk parameters in experimental hyperlipidemia. Lipids in Health and Disease. 2013; 12(1), pp.1-12.
27. Zhang J, Lian Q, Zhu G, Zhou F, Sui L, Tan C, et al.. A human iPSC model of Hutchinson Gilford Progeria reveals vascular smooth muscle and mesenchymal stem cell defects. Cell Stem Cell. 2011;8(1), pp.31-45.
28. Pinna C, Morazzoni P, Sala, A. Proanthocyanidins from Vitis vinifera inhibit oxidative stress-induced vascular impairment in pulmonary arteries from diabetic rats. Phytomedicine. 2017; 25, 39-44.
29. VaezMahdaviMR, RoghaniM, Baluchnejadmojarad T.. The role of nitric oxide and prostaglandins in the effects of alcoholic Trigonella foenum-graecum seed extract on aortic reactivity in streptozotocin-diabetic rats. Daru. 2008; 16(1), pp.29-34.
30. Srinivasan S, Hatley ME, Bolick DT, Palmer LA, Edelstein D, Brownlee M. et al. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. Diabetologia. 2004; 47(10), pp.1727-1734.
31. Ghareib SA, El-Bassossy HM, Elberry AA, Azhar A, Watson ML, Banjar ZM. 6-Gingerol alleviates exaggerated vasoconstriction in diabetic rat aorta through direct vasodilation and nitric oxide generation. Drug design, Development and Therapy. 2015; 9, 6019.
32. Harvey RD. Muscarinic receptor agonists and antagonists: Effects on cardiovascular function. Muscarinic Receptors. 2012; 299-316.
33. Cechinel-Zanchett CC, Tenfen A, Siebert DA, Micke G, Vitali L, Cechinel-Filho V, . Bauhinia forficata link, a Brazilian medicinal plant traditionally used to treat cardiovascular disorders, exerts endothelium-dependent and independent vasorelaxation in thoracic aorta of normotensive and hypertensive rats. Journal of Ethnopharmacology. 2019; 243, p.112118.
34. Hatake K, Wakabayashi I, Hishida, S. Endothelium-dependent relaxation resistant to NG-nitro-L-arginine in rat aorta. European Journal of Pharmacology. 1995; 274(1-3), pp.25-32.
35. Bryan RM, You J, Golding EM, Marrelli SP. Endothelium-derived hyperpolarizing factor: A cousin to nitric oxide and prostacyclin. The Journal of the American Society of Anesthesiologists. 2005; 102(6), pp.1261-1277.
36. Nelson MT, Quayle JM. Physiological roles and properties of potassium channels in arterial smooth muscle. American Journal of Physiology. 1995; 268, C799–C822.
37. Mistry DK, Garland CJ. Nitric oxide (NO)-induced activation of large conductance Ca^{2+}-dependent K+ channels (BKCa) in smooth muscle cells isolated from the rat mesenteric artery. British Journal of Pharmacology. 1998; 124(6), pp.1131-1140.
38. Karaki H, Ozaki H, Hori M, Mitsui-Saito M, Amano KI, Harada KI. et al. Calcium movements, distribution, and functions in smooth muscle. Pharmacological Reviews. 1997; 49(2), 157-230.
39. Chen GP, Ye Y, Li L, Yang Y, Qian AB, Hu SJ. Endothelium-independent vasorelaxant effect of sodium ferulate on rat thoracic aorta. Life Sciences. 2009; 84(3-4), 81-88.
40. Lee K, Jung J, Yang G, Ham I, Bu Y, Kim H, Choi HY. Endothelium-independent vasorelaxation effects of Sigesbeckia glabrescens (makino) makino on isolated rat thoracic aorta. Phytotherapy Research.

2013; 27(9), 1308-1312.
41. Guo J, Pereira TJ, Dalvi P, Yeung LSN, Swain N, Breen DM. et al. High-dose metformin (420 mg/kg daily po) increases insulin sensitivity but does not affect neointimal thickness in the rat carotid balloon injury model of restenosis. Metabolism. 2017; 68, 108-118.
42. Arshad R, and Karim N. Vasculo-Protective Cover: A Novel Action of Metformin. Journal of Bahria University Medical and Dental College. 2015; 5(3), 105-108.
43. Forouzandeh F, Salazar G, Patrushev N, Xiong S, Hilenski L, Fei, B. et al. Metformin beyond diabetes: pleiotropic benefits of metformin in attenuation of atherosclerosis. Journal of the American Heart Association. 2014; 3(6), e001202.
44. Romualdi D, Costantini B, Selvaggi L, Giuliani M, Cristello F, Macri F, et al. Metformin improves endothelial function in normoinsulinemic PCOS patients: A new prospective. Human Reproduction. 2008; 23(9), 2127-2133.
45. Matsumoto T, Noguchi E, Ishida K, Kobayashi T, Yamada N, Kamata K. Metformin normalizes endothelial function by suppressing vasoconstrictor prostanoids in mesenteric arteries from OLETF rats, a model of type 2 diabetes. American Journal of Physiology-Heart and Circulatory Physiology. 2008; 295(3), H1165-H1176.
46. Sartoretto JL, Melo GA, Carvalho MH, Nigro D, Passaglia RT, Scavone C, et al.. Metformin treatment restores the altered microvascular reactivity in neonatal streptozotocin-induced diabetic rats increasing NOS activity, but not NOS expression. Life Sciences. 2005; 77(21), pp.2676-2689.
47. Azemi AK, Mokhtar SS, Rasool AHG. Clinacanthus nutans leaves extract reverts endothelial dysfunction in type 2 diabetes rats by improving protein expression of eNOS. Oxidative Medicine and Cellular Longevity, 2020.
48. Majithiya JB, Balaraman R. Metformin reduces blood pressure and restores endothelial function in aorta of streptozotocin-induced diabetic rats. Life Sciences. 2006; 78(22), pp.2615-2624.
49. Zhao Z, Cheng X, Wang Y, Han R, Li L, Xiang T et al. Metformin inhibits the IL-6-induced epithelial-mesenchymal transition and lung adenocarcinoma growth and metastasis. PloS One. 2014; 9(4), e95884.
50. Dominguez LJ, Davidoff AJ, Srinivas PR, Standley PR, Walsh MF Sowers, J.R. Effects of metformin on tyrosine kinase activity, glucose transport, and intracellular calcium in rat vascular smooth muscle. Endocrinology. 1996; 137(1), pp.113-121.