The Vasorelaxant Effect of *Hibiscus sabdariffa* Linn. Polyphenol-Rich Extract (HPE) on Rat's Isolated Aorta

(Kesan Vasorelaksasi Ekstrak *Hibiscus sabdariffa* Linn. Kaya Polifenol (HPE) terhadap Aorta Terpencil Tikus)

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ABSTRACT

Hibiscus sabdariffa Linn. or also known as roselle which is rich in polyphenols, has been demonstrated to cause lowering of blood pressure in animal and clinical settings. However its exact mechanism of action particularly from polyphenolic compounds is not clearly understood. Therefore, we aimed to determine the effects of H. sabdariffa polyphenol extract (HPE) towards vascular reactivity and its mechanism of action. The HPE was studied on isolated thoracic aortic rings from normal Sprague-Dawley rats, suspended in a 15-ml organ chambers containing Krebs-Henseleit solution. The changes in tension were recorded by isometric transducer connected to data acquisition. HPE relaxed the contraction induced by phenylephrine (PE, 1 µM) in similar pattern for both endothelium-intact and endothelium denuded aortic rings in dose-dependent manner 0.1 ~ 0.9 mg/ml. The pretreatment with atropine (1 µM), a competitive muscarinic antagonist, and propranolol (1 µM), a non-selective beta- blocker did not alter HPE vasorelaxation response. In addition, HPE did not inhibit the contraction induced by extracellular Ca^{2+} precontracted by PE (1 µM) or KCl (60 mM), in Ca^{2+}-free solution, suggesting that the relaxation effect of HPE was not via inhibition of calcium channels. In conclusion, HPE demonstrated vasorelaxation effects on rat thoracic aorta although the underlying mechanism is still unknown. The vasorelaxation effect could be via angiotensin type 1 receptor inhibition in the vascular smooth muscle cells or the activation of hyperpolarizing K^+ channel.

Keywords: Hibiscus sabdariffa; polyphenolic extract; aorta; endothelium

ABSTRAK

Hibiscus sabdariffa L. atau dikenali sebagai rosel yang kaya dengan polifenol telah dibuktikan mampu menurunkan tekanan darah pada haiwan kajian dan ujian klinikal. Walau bagaimanapun, mekanisma sebenar daripada polifenol tersebut belum difahami sepenuhnya. Oleh itu, kajian ini dijalankan bagi mengkaji kesan ekstrak polifenol H. sabdariffa (HPE) ke atas reaktiviti vaskular dan mekanismanya. HPE diuji ke atas cecincin aorta toraks terpencil daripada tikus Sprague-Dawley normal yang disuspensi pada larutan Krebs-Henseleit. Perubahan pada ketegangan cecincin aorta direkodkan menggunakan tranduser isometrik. HPE memberi kesan relaksasi, secara kepekatan bersandar iaitu 0.1 ~ 0.9 mg/ml terhadap prakontraksi menggunakan fenilefrin (PE, 1 µM) pada cecincin aorta dengan kehadiran dan ketidakhadiran endotelium. Kesan relaksasi HPE pada cincin aorta tidak disekat pada rawatan awal menggunakan atropin (1 µM) iaitu antagonis kompetitif reseptor muskarinik, dan propranolol (1 µM) iaitu perencat reseptor adrenergik beta. Di samping itu, HPE tidak menghalang kontraksi yang diaruh dengan kemasukan ekstraselular Ca^{2+} ke dalam cincin aorta tikus yang diprakontraksi dengan PE (1 µM) atau KCl (60 mm) di dalam larutan Krebs-Henseleit tanpa Ca^{2+} dan ini mencadangkan kesan relaksasi HPE juga bukan melalui perencatan saluran kalsium. Kesimpulannya, HPE menghasilkan kesan relaksasi terhadap aorta toraks tikus namun mekanisma kesan relaksasi tersebut masih tidak diketahui. Kemungkinan mekanisma kesan relaksasi HPE adalah melalui aktiviti antagonistik pada reseptor angiotensin jenis 1 pada sel otot licin vaskular ataupun melalui pengaktifan saluran K^+ yang hiperpolarisasi.

Kata kunci: Hibiscus sabdariffa; ekstrak polifenol; vasorelaksasi; cecincin aorta; endotelium

INTRODUCTION

Hibiscus sabdariffa L. plant, better known as roselle is a native species of the family Malvaceae and is believed originated from West Africa. In Malaysia, roselle is also known as asam paya or asam susur because of its cranberry-like taste. Roselle is harvested for its calyces and consumed as a health drink (Esa et al. 2010).

There has been many studies conducted using extract of *Hibiscus sabdariffa* L. calyces. Its aqueous extract has been shown to possess anti-hypertensive and cardioprotective effects towards hypertension-induced rats (Ramalingam et al. 2016; Odigie et al. 2003) in dose-independent manners (Adegunloye et al. 2010). The possible mechanism of anti-hypertensive activities of aqueous and methanolic extracts of roselle on isolated aortic rings in hypertensive rats was reported to be most likely through the inhibition of angiotensin-converting enzyme activity (ACE) (Ajay et al. 2007), or calcium influx into vascular smooth muscle (VSM) as well as by the activation of endothelial nitric

oxide and cGMP (Mozaffari-Khosravil et al. 2009). In addition, roselle extract also possesses antioxidant activity which capable to protect low-density lipoproteins from oxidation (Hirunpanich et al. 2006). Gathering all these evidences, it is apparent that *H. sabdariffa* has the potential to be developed as a remedy for diseases associated with oxidative stress such as atherosclerosis, which ultimately can lead to many cardiovascular diseases.

Previous studies on *H. sabdariffa* polyphenol extract (HPE) have also been proven to possess antioxidant and hepatoprotective effects against in rats' liver (Adewale & Abiodun 2013). It was also reported that inhibition of lipopolysaccharide-induced inflammatory activity by HPE was through regulatory expression of cyclooxygenase-2 (COX-2) and its antioxidant effects (Kao et al. 2009). HPE was also found to inhibit hyperglycemia, hyperlipidemia and oxidative stress in addition to improved insulin resistance-induced in laboratory mice with Type-2 diabetes mellitus (T2DM) (Peng et al. 2011). However, there is not much research done on the effect of HPE especially within the cardiovascular system. Therefore, this study was conducted to investigate the effects of HPE towards vascular reactivity and its mechanism of action on the aorta.

MATERIALS AND METHODS

PREPARATION OF EXTRACTS

Roselle (*H. sabdariffa* Linn.) calyces were obtained from a plantation in Terengganu, Malaysia. Voucher specimens of *H. sabdariffa* calyces (UKMB 40308) were prepared and deposited in the Herbarium Universiti Kebangsaan Malaysia (Bangi, Malaysia). The HPE was prepared as previously described (Lim et al. 2016). About 5 g of dried UKMR-1 roselle calyces were crushed in a blender. The powdered calyces were extracted with 50 mL of an HPLC-grade methanol and stirred in a 60°C water bath for 30 minutes. The extract was then filtered through a Whatman No. 4 filter paper, and the remaining residues were extracted for two more times. The extract was then pooled and concentrated using rotary evaporator ($\leq$ 5°C, 20 mbar). The residue was then solubilized in 10 mL of deionized water (pH 2.3). The aqueous solution was then re-extracted with hexane (to remove pigment) (3×10 mL) followed by ethyl acetate (3×10 mL). The ethyl acetate soluble fraction was then allowed to evaporate to dryness and the dried crude HPE was stored at -40°C for further usage.

PHYTOCHEMICAL SCREENING

Total phenol content was estimated using Folin-Ciocalteau reagent based assay as previously described (Velioglu et al. 1998). The aluminum chloride colorimetric method was used to measure the flavonoid content in HPE as described by Peng et al. (2011).

DRUGS AND CHEMICALS

The following chemicals were obtained from the sources specified: phenylephrine, acetylcholine, atropine and propranolol (Sigma-Aldrich, St. Louis, USA). All drugs were dissolved in distilled water.

EXPERIMENTAL ANIMALS

Eighteen male *Sprague-Dawley* rats weighing 250-350 grams were obtained from the Laboratory Animal Resource Unit (LARU) Universiti Kebangsaan Malaysia (UKM). All animal experimental protocols were performed in accordance with the guidelines issued by the Universiti Kebangsaan Malaysia Animal Ethics Committee. Rats were housed in clean cages in an air-conditioned room (24 ±2°C and 40 ± 5% humidity) on a day/night and fed with rat chow and water *ad libitium* throughout the experimental period.

PREPARATION OF RAT THORACIC AORTA RINGS

The rats were sacrificed and the thoracic aorta was carefully isolated, cleaned from the adherent fat and connective tissue and was then cut into 3-5 mm rings. The rings were suspended horizontally in tissue chambers containing 10 mL of Krebs-Henseleit solution (KHS) of following composition (in g): NaCl (6.95), NaHCO$_3$ (2.10), MgSO$_4$.7H$_2$O (0.29), KCl (0.35), KH$_2$PO$_4$ (0.14), glucose (2.00), and CaCl$_2$.2H$_2$O (0.35). Careful handling was also taken to avoid damage to the endothelium. The tissue-bath solution was aerated continuously with 95% O$_2$–5% CO$_2$ at 37°C. Aortic rings were allowed to equilibrate at an optimal tension of 1 g and if needed, the tension was readjusted to 1 g. Responses were recorded isometrically via a force–displacement transducer connected to a PowerLab 4/30 recording system (AD Instruments, Sydney, Australia) which was equipped with a display monitor.

PHARMACOLOGICAL STUDIES

At the beginning of the experiment, the viability of the aortic ring was tested by repeated exposure to KCl solution (high K$^+$, 80 mM). Then, the presence of intact endothelial cells were confirmed by pre-contracting the issue with PE (1 µM) and followed by relaxation with ACh (1 µM). Relaxation of no less than 60% indicated the presence of intact endothelial cells. For the vasorelaxation study, the rings were pre-contracted with α$_1$- agonist phenylephrine (PE, 1 µM), and the relaxant effects of HPE at different concentrations (0.1 ~ 0.9 mg/mL) were recorded at 3 min intervals between successive concentrations. The relaxation effects of HPE was recorded in the two different preparations, ie. intact and denude endothelium. Denuded endothelium rings were obtained by gently rubbing the intimal surface of the tissue with blunted forceps. The endothelium was considered as denuded when there was less than 10%

relaxation in response to ACh (1 μM). To study the possible involvement of cholinergic and β-adrenergic receptors, relaxations of HPE was performed in pre-incubated rings with atropine (1 μM), a competitive muscarinic antagonist and propranolol (1 μM) a non-selective β-blocker. To investigate the effects of HPE on extracellular Ca^{2+}- induced contractions, two sets of experiments were conducted: (1) evaluation of receptor-operative voltage dependent Ca^{2+} channels (ROCCs) and (2) evaluation of voltage-dependent Ca^{2+} channels (VDCCs). In the experiment for ROCCs, the contractile responses induced by $CaCl_2$ (0.3 – 10 mM) in endothelium-denuded aortic rings were contracted by PE (1 μM) in Ca^{2+}- free KHS, with and without a 10-minute pre-incubation with HPE (0.9 mg/ml). The experiment for VDCCs was conducted under the same procedure, except that contraction was induced by KCl (60mM).

DATA PRESENTATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using independent t test, one way ANOVA and one-way repeated measure ANOVA test. The significant levels of the data were determined at $p < 0.05$. All values are given as mean ± S.E.M.

RESULTS

PHYTOCHEMICAL SCREENING

The extraction yield, total phenolic content and flavonoid content obtained in HPE is shown in Table 1.

TABLE 1. The extraction yield, total phenolic content and flavonoid content obtained for HPE

Total Extraction Yield (g extract)	0.43
Total Phenolic Content (mg GAE/g extract)	65.02 ± 7.15
Relative Percentage of Phenolic in HPE (%/g extract)	15.12 ± 0.79
Total Flavonoid Content (mg QrE/g extract)	10.95 ± 0.84
Relative Percentage of Flavonoid in HPE (%/g extract)	2.55 ± 0.39

HPE CAUSES VASORELAXATION

There was no significant difference ($p > 0.05$) between the vasorelaxation response of endothelium-intact and endothelium-denuded to all HPE concentrations (Figure 1A). An example of vasorelaxation tracing of HPE in dose-dependent manner with concentration range of 0.1-0.9 mg/ml is shown in Figure 1B.

EFFECTS OF HPE RELAXATION ON CHOLINERGIC AND ADRENERGIC RECEPTORS

There were no alteration on the vasorelaxant activity of HPE on aortic rings pre-incubated with atropine (1 μM) (Figure 2) or propranolol (1 μM) (Figure 3). There was no significant difference ($p > 0.05$) between the relaxation

A
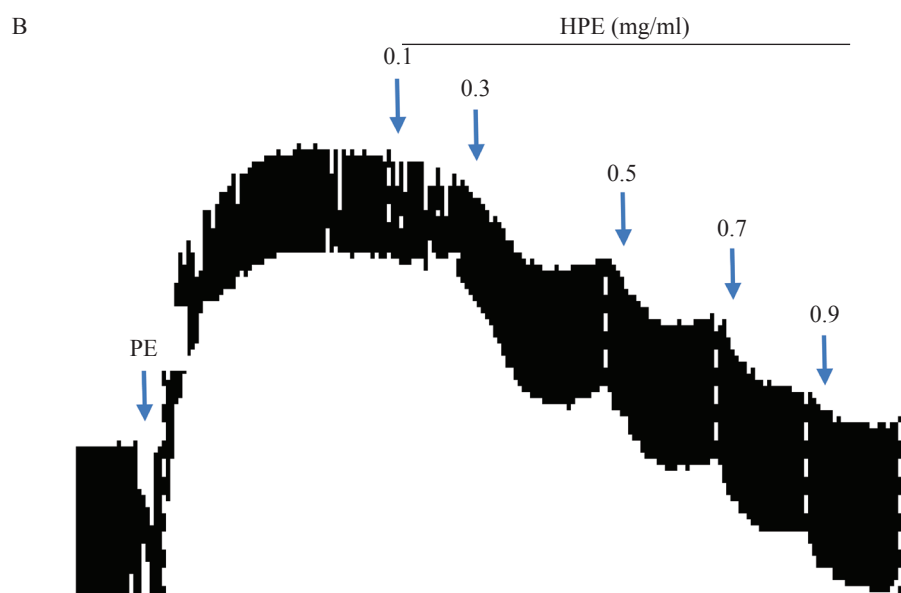

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B
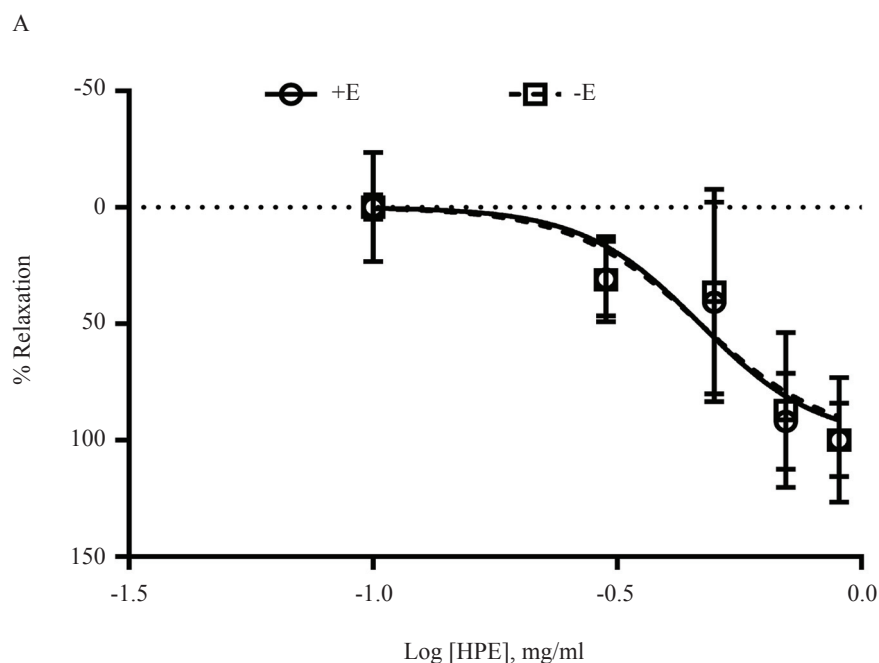
FIGURE 1. [A] Concentration-dependent relaxant effects of HPE (0.1 ~ 0.9 mg/ml) on phenylephrine (PE, 1 µM)-pre-contracted rat aortic rings with [(+) Endo] or without [(-) Endo] endothelium in Krebs-Henseleit solution. [B] An example of HPE-induced relaxation tracing in dose-dependent manner. Values are given as mean ± S.E.M.
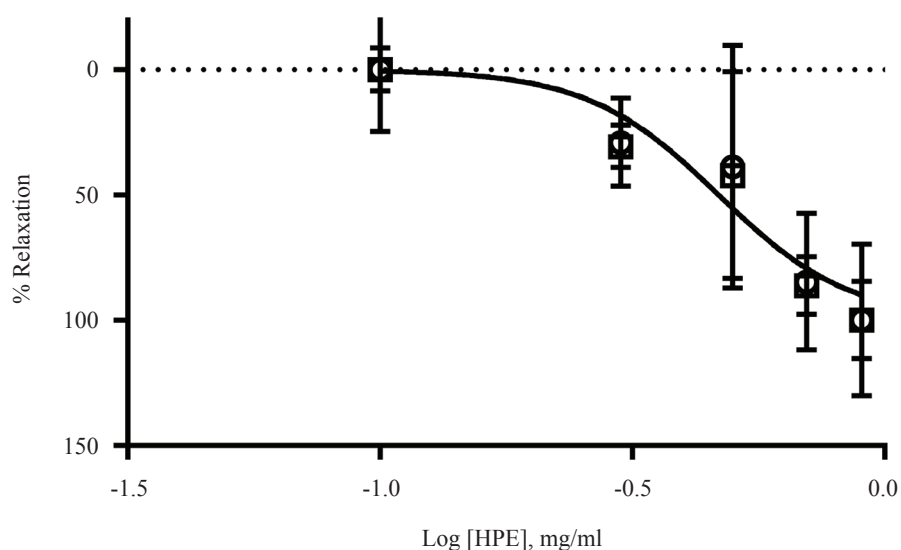

FIGURE 4. Effects of HPE against phenylephrine (PE, 1µM)-induced contractions in endothelium-intact aortic rings incubated with atropine (1µM). Relaxation was calculated as % reduction in the respective contraction. Values are given as mean ± S.E.M.

responses of HPE with and without the pre-incubation of atropine or propranolol. Two indicate that the relaxant induced by HPE was independent of both cholinergic and adrenergic receptors on vascular smooth muscle.

EFFECTS OF HPE ON EXTRACELLULAR CA²⁺ -INDUCED CONTRACTION

Pre-incubation of the aortic rings with 0.9 mg/ml HPE were not able to inhibit the contraction of aortic rings induced by PE (1 µM) (Figure 4A) or KCl (60 mM) (Figure 4B) followed by extracellular Ca^{2+} addition in the Ca^{2+}-free Krebs solution. There was no significant difference ($p > 0.05$) between vasoconstriction responses of the aortic

rings with or without the pre-incubation of HPE. This data suggesting that, vasorelaxation responses of HPE was not mediated by the inhibition of extracellular Ca^{2+} influx on vascular smooth muscle either in ROCC (PE- induced) or VDCC (KCl-induced).

DISCUSSION

Development of dietary polyphenols as functional food could contribute to prevent and improve cardiovascular diseases risk. Our previous finding has shown that HPE contains various polyphenols which are beneficial for cardiovascular diseases, ie. anthocyanin, chlorogenic acid,
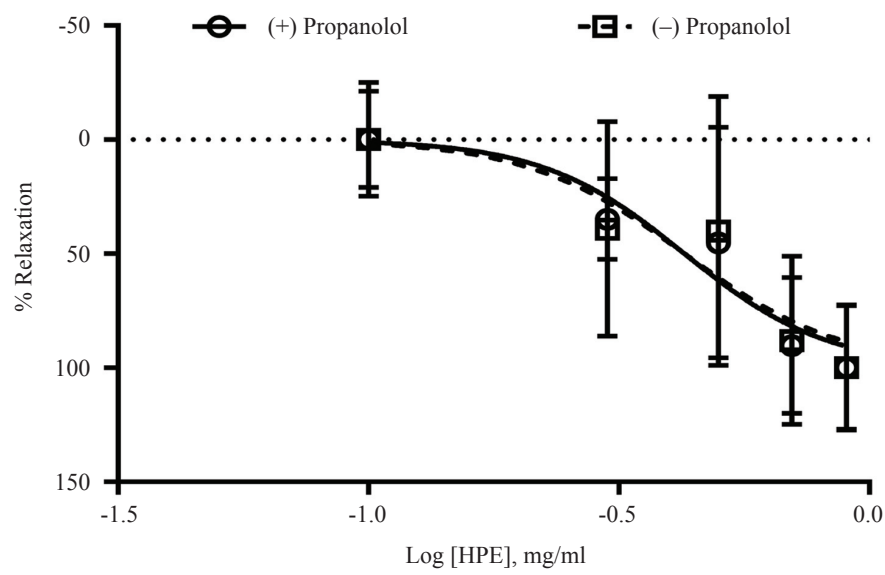
FIGURE 3. Effects of HPE against phenylephrine (PE, 1 μM)-induced contractions in endothelium-intact aortic rings incubated with propranolol (1 μM). Relaxation was calculated as % reduction in the respective contraction. Values are given as mean ± S.E.M.
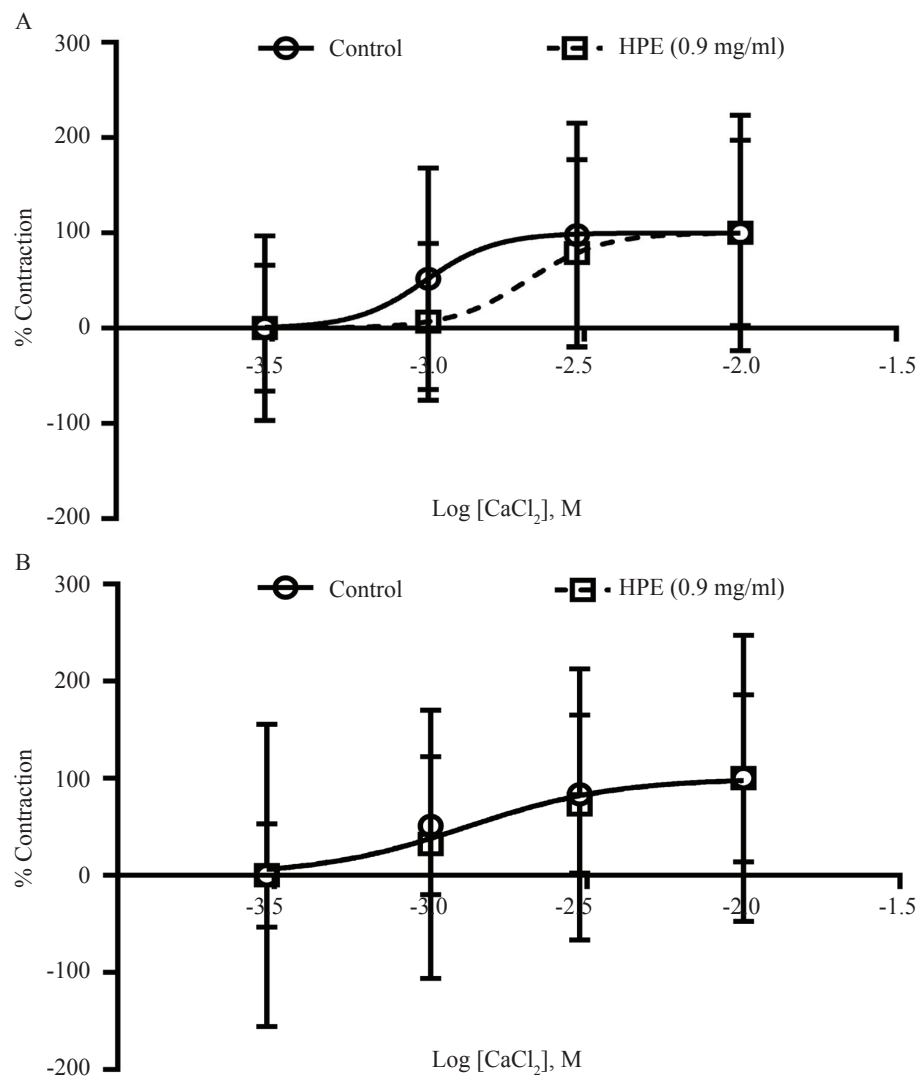

FIGURE 4. Inhibitory effect of HPE (0.9 mg/ml) on the contraction induced by extracellular Ca^{2+} addition (0.3-10 mM) in endothelium-denuded rat aortic rings pre-contracted by phenlyephrine (PE, 1 μM) [A] or KCl (60 mM) [B] in Ca^{2+}-free KHS. Values are given as mean ± S.E.M.

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quercetin and gallic acid (Lim et al. 2016). In this study, we have shown that HPE managed to induce relaxation in the aortic rings regardless of whether the endothelium was intact or denuded. The endothelium is an important component in the vessels because it is responsible for the secretion of endothelium-derived relaxing factor (EDRF), namely nitric oxide (NO) (Stankevicius et al. 2003). It was reported that, the EDRF-induced vasorelaxation was due to rapid increase in cyclic GMP levels in vascular smooth muscle (VSM) (Ignarro et al. 1989) and gallic acid was found to increase such NO levels via endothelial nitric oxide synthase (eNOS) in HuVECs experiment (Kang et al. 2015). In contrast to our findings, HPE relaxation was independent to the endothelium. Possibly, HPE exerts direct action to the vascular smooth muscle as it was shown in quercetin previously (Perez-Vizcaino et al. 2002). They speculated that vasodilation may result from inhibition of protein kinases involved in the Ca^{2+} sensitizing mechanisms responsible for smooth muscle contraction.

Vascular smooth muscle (VSM) relaxation can also be triggered by the stimulation of the M_3 muscarinic receptor subtype on blood vessels despite the lack of parasympathetic innervation in the blood vessels (Brunning et al. 1994). M_3 muscarinic receptor subtype is located on the vascular endothelial cells and stimulation of these receptors causes the release of EDRF (McFadzean & Gibson 2002) such as nitric oxide (NO), which will diffuse into VSM cells and cause relaxation (Stankevicius et al. 2003). The findings of our study suggested that there was no direct involvement of the M_3 muscarinic receptor subtype on HPE relaxing effect as there was no significant difference in the HPE-induced relaxation in the presence and absence of atropine, a competitive antagonist to the muscarinic receptor. On the contrary, previous study has shown that *H. sabdariffa* methanolic extract induced significant relaxation effects on thoracic aorta were incubated with atropine compared with control, but only at the highest concentration of extract which was (1 mg/ml) (Ajay et al. 2007).

To investigate the possible involvement of HPE vasorelaxant effect on VSM through antagonistic activity of β-adrenergic receptors, the vasorelaxant effects of HPE were examined via incubation of the aortic rings with propranolol, a non-selective β-blocker. In the presence of propranolol, there was no attenuation of the relaxation effect of HPE in any concentration. Hence, our study suggested that, β-adrenergic receptor was not involved in the HPE-induced relaxation.

The presence of high amount of Ca^{2+} in the intracellular VSM is a major factor for its contraction. There are several influx pathways of Ca^{2+} into intracellular VSM namely, receptor operated calcium channel (ROCCs) (McFadzean & Gibson 2002) and voltage dependent calcium channel (VDCCs) (Nelson & Quayle 1995). The binding of agonist such as phenylephrine (PE) to the receptors will further activate ROCCs thus result in the influx of Ca^{2+} into intracellular VSM and cause contraction. Meanwhile, the depolarization of VSM cell membrane by the presence of high K^+ will activate VDCCs and cause influx Ca^{2+} thus produce VSM contraction. Our results showed that, the relaxation effects of HPE were not dependent on either the inhibition of calcium channel operated by receptors or voltage.

Overall, our present study has also demonstrated that HPE could induce vasodilatory effects in isolated aortas from normal rats independent of endothelium. The endothelium-dependent vasodilatory effects of HPE might be via endothelium histamine H_1 receptors through mediation of EDRF(s) other than NO, whereas endothelium-independent vasodilatory effects most likely through the activation of voltage dependent potassium ion (K^+) channels on VSM. Opening of the voltage channels generates the outflow K^+ out of VSM cells which hyperpolarize the membrane and invoked the closures of voltage dependent Ca^{2+} channel which resulted in the vasorelaxation, as it has been shown in red wine polyphenols (Ndiaye et al. 2003).

CONCLUSION

The finding of the present study shows that *Hibiscus sabdariffa* L. polyphenol extract are able to induce vasorelaxant effect. However, the exact mechanism of relaxation effects induced by HPE is remain unknown since it shows an independent relaxation responses to cholinergic and adrenergic receptors on the vascular smooth muscle. Besides, HPE was not able to inhibit the extracellular Ca^{2+} influx in vascular smooth muscle via both receptor-dependent Ca^{2+} channel (ROCC) and voltage-dependent Ca^{2+} channel (VDCC). Therefore, the relaxant responses by HPE was probably mediated by other mechanism such as the involvement of type-I angiotensin receptor, histamine receptor or probably K^+ channel. Therefore, further study are essential to find out the underlying mechanisms involved. Nevertheless, these findings provide new understanding towards the potential of polyphenolics extracts of *Hibiscus sabdariffa* as a vasorelaxant agent for development of alternative adjuvant in preventing hypertension as well as other cardiovascular diseases.

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