Research Article

Vascular Reactivity Concerning Orthosiphon stamineus Benth-Mediated Antihypertensive in Aortic Rings of Spontaneously Hypertensive Rats

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1. Introduction

Orthosiphon stamineus Benth has been traditionally used to treat hypertension. The study aimed to investigate the vascular reactivity of water extract (WOS) and water:methanolic (1:1) extract (WMOS) of Orthosiphon stamineus Benth and AT1 receptors blocker in the mechanisms of antihypertensive mediated by α1-adrenergic receptor and EDNO and PGI2 releases in the SHR aortic rings. SHR (230–280 g) were divided into four groups: control, WOS, WMOS, and losartan. After being fed orally for 14 days, the aorta was harvested and subjected to PE (10^-9 to 10^-5 M) and ACh (10^-9 to 10^-5 M) with and without L-NAME (100 μM) and indomethacin (10 μM), respectively. WOS, WMOS, and losartan significantly reduced the contractile responses to PE intact suggesting the importance of endothelium in vasorelaxation. Losartan significantly enhanced the ACh-induced vasorelaxation. L-NAME significantly inhibited the ACh-induced relaxation in all groups. Indomethacin enhanced ACh-induced vasorelaxation in WMOS. Collectively, Orthosiphon stamineus leaves extract reduced vasoconstriction responses by the alteration of α1-adrenergic and AT1 receptors activities. The involvement of EDNO releases was clearly observed in this plant. In WOS, PGI2 releases might not participate in the ACh-induced vasorelaxation. However, in WMOS, enhancement of vasorelaxation possibly due to continuous release of PGI2.

Orthosiphon stamineus leaves extracts have been used as traditional medicine [2] and possess benefits such as antidiabetic, ability to increase plasma triglyceride and plasma HDL-cholesterol concentrations [3], anti-lithiatic and hypouricemic effects [4, 5], antifungal [6], and ability to treat kidney stone and urinary tract diseases [7–9]. It has traditionally been used in Java for the treatment of hypertension and diabetes [1]. Hypertension has been reported to be associated with endothelium dysfunction in both human and animal studies [10]. Endothelium regulates vascular tone by releasing vasoconstrictors such as endothelins, prostanoids and oxygen reactive species, and vasodilators such as nitric oxide (NO), prostacyclin (PGI2), and endothelial hyperpolarizing factor (EDHF). These vasodilators were a great discovery by Furchgott and Zawadzki in 1980 [11], known as endothelium derived relaxing factors (EDRF). They have been reported by Peach et al. [12] that releases of EDRF caused vasorelaxant effects of acetylcholine (ACh), which is dependent on the presence of the endothelial cells [11, 13].

Phenylephrine (PE) is a selective α1-adrenergic receptor agonist that increases arterial blood pressure by peripheral vasoconstriction. α1-Adrenergic receptors which exist
postsynaptically are G-protein-coupled receptors, and thus activation of a cellular signaling is subsequent to the interaction with a G-protein. Activation of these receptors on vascular smooth muscle leads to vasoconstriction. PE has predominantly \( \alpha_1 \) postsynaptic receptors in rat's aorta [14]. Since PE is a selective \( \alpha_1 \)-adrenergic receptor agonist and losartan is AT\(_1\) receptor blocker, there is possible relationship between AT\(_1\) and \( \alpha_1 \) receptors [15]. In addition, crosstalk between AT\(_1\) and \( \alpha_1 \) receptors in the smooth muscle of rabbit aorta is endothelium dependent [16]. It has been reported that MRC A isolated from *Orthosiphon stamineus* causes continuous decreases in systolic blood pressure (SBP) and heart rate (HR) after subcutaneous administration in conscious SHR [17]. However, studies on the antihypertensive mechanisms by *Orthosiphon stamineus* still remain unclear. The present study aimed to investigate the vascular reactivity of water extract (WOS) and water:methanolic (1:1) extract (WMOS) of *Orthosiphon stamineus* Benth and AT\(_1\) receptors blocker in the mechanisms of antihypertensive effects mediated by \( \alpha_1 \)-adrenergic receptor and prostacyclin (PGI\(_2\)) and endothelium-derived nitric oxide (EDNO) releases in the SHR aortic rings.

### 2. Materials and Methods

#### 2.1. Preparation of *Orthosiphon stamineus* Leaves Extracts.

Voucher specimen (no. 11009) of the plant material was deposited at Herbal Room, School of Pharmaceutical Sciences, Universiti Sains Malaysia (USM). WMOS was prepared by having dried and ground *Orthosiphon stamineus* leaves extracted by a mixture of methanol: water (1:1) using a Soxhlet extractor for a period of 12 hours, whereas preparation of WOS involved hot maceration of the dried and ground *Orthosiphon stamineus* leaves at 50°C for 6 hours and was repeated thrice. Each extract was bulked and concentrated in a rotary evaporator under vacuum and then freeze-dried were less than 10% relaxations to ACh (10\(^{-5}\) M). Relaxation not less than 60% indicated the presence of intact endothelial cells. Responses were recorded isometrically via a force transducer (Grass FT03D) connected to a computerized data acquisition system (PowerLab; ADInstruments Pty Ltd., Australia). For vasoconstriction study, the concentration-response curves for PE (cumulative final concentration of \( 10^{-9} \) to \( 10^{-5} \) M) were recorded. The contraction effects of PE were recorded in two different preparations, intact and denude endothelium. Denude endothelium of aortic rings was obtained by gently rubbing the intimal layer of the tissue with a blunt needle for a few times. The aortic rings were considered denuded when there were less than 10% relaxations to ACh (1 \( \mu \)M) precontracted with PE (1 \( \mu \)M) whereas in order to obtain the concentration-response curves of relaxation, ACh (\( 10^{-9} \) to \( 10^{-5} \) M) was added cumulatively to the chamber at the plateau of the PE (1 \( \mu \)M) precontracted aortic rings at 3-minute intervals. To further assess the involvement of EDNO and prostacyclin (PGI\(_2\)) releases, relaxations of aortic rings were performed in WOS, WMOS, and losartan groups preincubated for 30 minutes with L-NAME (100 \( \mu \)M), a nonspecific NO synthase inhibitor, and indomethacin (10 \( \mu \)M), a nonspecific cyclooxygenase inhibitor, respectively.

#### 2.2. Animals.

Male spontaneously hypertensive rats (SHR, 230–280 g) were housed in individual cages with free access to foods and water and maintained at Animal Transit Facility of School of Pharmaceutical Sciences, USM. All procedures involving animals were conducted according to the ethical guidelines by the Animal Ethics Committee, USM. The animals were divided into four groups: (1) WOS, 1000 mg/kg; (2) WMOS, 1000 mg/kg; (3) losartan, 10 mg/kg; and (4) control (vehicle). All animals were given daily treatment orally for 14 days before being subjected to vascular reactivity studies.

#### 2.3. Drugs and Chemicals.

Phenylephrine hydrochloride (PE), acetylcholine (ACh), indomethacin, and \( N^\circ \)-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma-Aldrich, Germany, while sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH\(_2\)PO\(_4\)), magnesium sulphate (MgSO\(_4\)-7H\(_2\)O), glucose, sodium hydrogen carbonate (NaHCO\(_3\)), and calcium chloride dehydrate (CaCl\(_2\)-2H\(_2\)O) were purchased from R&M Chem., UK. All drugs were freshly prepared in normal saline, except indomethacin in 0.5% (w/v) sodium carbonate, prior to use.

#### 2.4. Vascular Reactivity Using Aortic Rings.

The rat was anesthetized with sodium pentobarbital (60 mg/kg, i.p.). A midline abdominal incision was performed to expose the aorta. The thoracic aorta was carefully isolated, cleaned from the adherent fat and connective tissues, and cut into 3–5 mm rings. The aortic rings were then suspended horizontally in tissue chambers containing 10 mL of Kreb’s solution (mmol/L: NaCl 118.6, KCl 4.8, CaCl\(_2\) 2.5, MgSO\(_4\)-7H\(_2\)O 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25.1, and glucose 11.0). The tissue-bath solution was bubbled incessantly with 95% O\(_2\) and 5% CO\(_2\) (carbogen) at 37°C. Aortic rings were then allowed to equilibrate at an optimal tension of 1 g for 45 min. Kreb’s solution was replaced every 15 min, and the tension was readjusted to 1 g when necessary. At the beginning of the experiment, the presence of intact endothelial cells was confirmed by precontracting the tissues with PE (1 \( \mu \)M) and followed by relaxation with ACh (1 \( \mu \)M). Relaxation not less than 60% indicated the presence of intact endothelial cells. Responses were recorded isometrically via a force transducer (Grass FT03D) connected to a computerized data acquisition system (PowerLab; ADInstruments Pty Ltd., Australia). For vasoconstriction study, the concentration-response curves for PE (cumulative final concentration of \( 10^{-9} \) to \( 10^{-5} \) M) were recorded. The contraction effects of PE were recorded in two different preparations, intact and denude endothelium. Denude endothelium of aortic rings was obtained by gently rubbing the intimal layer of the tissue with a blunt needle for a few times. The aortic rings were considered denuded when there were less than 10% relaxations to ACh (1 \( \mu \)M) precontracted with PE (1 \( \mu \)M) whereas in order to obtain the concentration-response curves of relaxation, ACh (\( 10^{-9} \) to \( 10^{-5} \) M) was added cumulatively to the chamber at the plateau of the PE (1 \( \mu \)M) precontracted aortic rings at 3-minute intervals. To further assess the involvement of EDNO and prostacyclin (PGI\(_2\)) releases, relaxations of aortic rings were performed in WOS, WMOS, and losartan groups preincubated for 30 minutes with L-NAME (100 \( \mu \)M), a nonspecific NO synthase inhibitor, and indomethacin (10 \( \mu \)M), a nonspecific cyclooxygenase inhibitor, respectively.

#### 2.5. Data Analysis.

All data are given as mean ± standard error means (SEM). PE-induced contraction and ACh-induced relaxation were analysed using one-way ANOVA followed by Dunnett's post hoc test, whereas the effects of ACh-induced relaxation after preincubated by L-NAME and indomethacin were analysed using Student's t-test. \( E_{\text{max}} \), \( R_{\text{max}} \), and \( pD_2 \) values were derived from nonlinear regression analysis. All analyses were using the computer software GraphPad Prism 5.0 for Windows (GraphPad Software Inc., USA). Values of \( P < 0.05 \) were considered statistically significant.
3. Results

3.1. Vasoconstriction Effects of PE on Aortic Rings. Cumulative additions of PE (10^{-9} to 10^{-5} M) produced a concentration-dependent contraction of aortic rings in all groups. In PE intact, WOS, WMOS, and losartan significantly decreased ($P < 0.05$) the contractile responses as compared to control whereas, in PE denude endothelium, no significant changes were obtained (Figure 1). Maximal contractile responses ($E_{max}$) in PE intact were significantly decreased in WOS, WMOS, and losartan (0.30 ± 0.06, 0.33 ± 0.03, 0.35 ± 0.03 versus 0.90 ± 0.10). In contrast, the $E_{max}$ of WMOS significantly enhanced the contraction responses in PE denude (0.57 ± 0.02 versus 0.43 ± 0.04). The pD$_2$ values from both PE intact and denude endothelium were unaltered as shown in Table 1.

3.2. Vasorelaxant Effects of ACh Precontracted with PE on Aortic Rings. ACh (10^{-9} to 10^{-5} M) produced dose-dependent relaxation in all groups in aortic rings precontracted with PE (1 M). Only losartan significantly enhanced ($P < 0.05$) the relaxant effect of ACh as compared to control ($R_{max}$ 111.20 ± 4.08 versus 73.15 ± 3.03). Both extract groups did not significantly alter the vasorelaxant effects of ACh as shown in Figure 2 and Table 2.

3.3. Effects of L-NAME on ACh-Induced Relaxation in Aortic Rings in WOS, WMOS, and Losartan Groups. To assess the contribution of EDNO, the aortic rings were preincubated with L-NAME (100 M), a NO synthase inhibitor for 30 minutes. ACh-induced relaxations in all groups were significantly inhibited ($P < 0.05$) by L-NAME as shown in Figure 3. $R_{max}$ and pD$_2$ values were tabulated in Table 2.

3.4. Effects of Indomethacin on ACh-Induced Relaxation in Aortic Rings in WOS, WMOS, and Losartan Groups. To investigate the role of prostacyclin (PGI$_2$) release, the aortic rings were preincubated with indomethacin (10 M), a COX inhibitor for 30 minutes. Indomethacin significantly reduced ($P < 0.05$) the ACh-induced relaxations in losartan and in contrast, significantly improved vasorelaxation in WMOS (Figure 4). $R_{max}$ and pD$_2$ values were tabulated in Table 2.

3.5. Role of Intracellular and Extracellular Calcium Mobilization on the PE-Induced Contraction. To assess the role of intracellular and extracellular calcium mobilization, the aortic rings were incubated in Ca$^{2+}$-free medium containing...
The present study demonstrated that, in intact endothelium, the contractile response to phenylephrine (PE), a selective agonist for \( \alpha_1 \)-adrenergic receptor, was significantly lowered in SHR treated with WOS and WMOS as compared to control. No significant change was seen in denude endothelium. These results showed that 14-day oral treatment of WOS and WMOS affected the \( \alpha_1 \)-adrenergic receptors activities in this preparation. The use of PE as a vasoconstrictor in the present study because the rat’s aorta has predominantly \( \alpha_1 \)-postjunctional receptors [14]. Furthermore Griffith et al. [19] and Martin et al. [20] demonstrated that suppression of constrictor responses to several agonists such as PE in the intact vascular endothelium may be due continuously basal release of endothelium-derived relaxing factor (EDRF) from endothelial cells. As seen in the present study, WOS and WMOS inhibited the contraction induced by PE as comparable to losartan. There were studies found that possible crosstalk between \( \alpha_1 \) and \( \alpha_1 \)-adrenoceptors existed [21–23]. Furthermore, Maeso et al. [24] reported that losartan reduced vasoconstrictor responses to PE in SHR aortic rings via endogenous Angiotensin II (AngII) acting on \( \alpha_1 \) receptors. Activation of \( \alpha_1 \) receptors results in increasing systolic blood pressure (SBP), blood vessels growth, and associated vascular smooth muscle cells (vsmc) apoptosis [25]. Blockade of \( \alpha_1 \) receptors which inhibit the effects of AngII may promote good prognosis in pathological conditions such as hypertension and to inhibit vasoconstriction [26]. Hence, we may suggest that (1) Orthosiphon stamineus leaves extracts exert their antihypertensive effects by blunting the increase of blood pressure in SHR; and (2) Orthosiphon stamineus leaves extracts may play their role in reducing vasoconstriction similar as \( \alpha_1 \) receptor blocker. WOS and WMOS may possibly possess antihypertensive properties and exert similar effects through these interactions.

Our results showed that the presence of endothelium is very important in vasorelaxation. It is likely that contribution by the variable EDRF such as NO, prostacyclin, and EDHF caused vascular smooth muscle cells to relax. The necessary endothelial cells for the relaxation by acetylcholine (ACh) to be occurred have been discovered since 1980 by Furchgott and Zawadzki. They demonstrated that loss of endothelium by rubbing the intimal surface of aorta caused no relaxation by the variable EDRF such as NO, prostacyclin, and EDHF. Furthermore, Maeso et al. [24] reported that losartan reduced vasoconstrictor responses to PE in SHR aortic rings via endogenous Angiotensin II (AngII) acting on \( \alpha_1 \) receptors. Activation of \( \alpha_1 \) receptors results in increasing systolic blood pressure (SBP), blood vessels growth, and associated vascular smooth muscle cells (vsmc) apoptosis [25]. Blockade of \( \alpha_1 \) receptors which inhibit the effects of AngII may promote good prognosis in pathological conditions such as hypertension and to inhibit vasoconstriction [26]. Hence, we may suggest that (1) Orthosiphon stamineus leaves extracts exert their antihypertensive effects by blunting the increase of blood pressure in SHR; and (2) Orthosiphon stamineus leaves extracts may play their role in reducing vasoconstriction similar as \( \alpha_1 \) receptor blocker. WOS and WMOS may possibly possess antihypertensive properties and exert similar effects through these interactions.

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the aortic rings isolated from these rats showed no effect to the ACh-induced relaxation. In contrast, losartan showed significantly greater relaxation. It is plausible that blockade of AT$_1$ receptor further enhanced the relaxation caused by ACh. Furthermore, Schiffrin and Touyz [27] demonstrated that losartan enhanced the endothelium-dependent relaxation to ACh in SHR aortic rings. In this preparation, the relaxation to ACh was probably due to the production or release of EDRF [12] and the endothelial vasorelaxant factors derived from cyclooxygenase (COX) pathways (prostacyclin PGI$_2$ released from endothelial cells).

WOS showed similar result of ACh-induced relaxation after preincubation with indomethacin. This similar effect has been reported by Luscher and Vanhoutte [28]. However, WMOS improved vasorelaxation to ACh after blockade of COX pathways. In this case, there was plausibility because the vasodilator PGI$_2$ was continuously released as indicated by its tonic effects on platelet cyclic adenosine monophosphate (cAMP) [29]. In contrast, losartan significantly reduced the ACh-induced relaxations, which may be due to the attenuation of PGI$_2$ production which was compensated for by the enhanced release of another vasodilator, for example, nitric oxide (NO). In this point of view, we might suggest that WMOS and blockade of AT$_1$ receptors modulate the derived endothelial vasorelaxant factors such as PGI$_2$ from COX pathways.

The release of NO by endothelial cells to vascular smooth muscle cells causes vasorelaxation. NO to play a vital role in the maintenance of vascular tone [30]. In order to assess the contribution of NO releases in the vasorelaxant effects elicited by Orthosiphon stamineus leaves extracts, we preincubated the rat aortic rings with L-NAME (100 μM), NO synthase inhibitor. Our study showed that the ACh-induced vasorelaxation in all treatment groups reduced significantly after

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**Figure 3:** ACh-induced relaxation responses of aortic rings precontracted with PE (1 μM) in the presence of L-NAME (100 μM) in control (vehicle) (a), losartan (10 mg/kg) (b), WOS (1000 mg/kg) (c), and WMOS (1000 mg/kg) (d). Responses to ACh are expressed as percentage of relaxation. Values are mean ± SEM of 5 to 6 SHRs in each group. *Denotes $P < 0.05$ compared to ACh without L-NAME (■).
Inhibition of NO synthase pathways. From the present data, it could be clearly proven that NO synthase pathways were involved in the vasorelaxation in the SHR.

In view of the present study, it is plausible that the vasorelaxant activities produced by *Orthosiphon stamineus* extracts may take place in the vascular smooth muscle cells. To investigate the effects of both extracts and losartan on the role intracellular Ca\(^{2+}\) on the contractility of the vascular smooth muscle cells of the aortic rings, media absent of and with Ca\(^{2+}\) were used. Significantly reduced contraction response to PE in denude aortic rings observed in the WOS and losartan were possibly due to inhibition of intracellular Ca\(^{2+}\) release from the sarcoplasmic reticulum at the level of vascular smooth muscle cells. Decreased intracellular Ca\(^{2+}\) concentration and increased myosin light chain phosphatase activity may had caused the smooth muscle to undergo weaker vascular contractility. Also, inhibition of receptor- and voltage-operated Ca\(^{2+}\) channels in the plasma membrane reduced Ca\(^{2+}\) influx may contribute as well [31].

5. Summary

In conclusion, our studies showed that water extract (WOS) and water : methanolic (1:1) extract (WMOS) of *Orthosiphon stamineus* Benth leaves promote antihypertensive effects by reducing vasoconstriction through the alteration of \(\alpha_1\)-adrenergic and \(AT_1\) receptors activities. Vasorelaxant effects of both WOS and WMOS may possibly involve mainly the release of EDNO. In WOS, PGI\(_2\) releases might not be participated in the ACh-induced vasorelaxation. However
in WMOS, enhancement of vasorelaxation might be due to the fact that vasodilator PGI$_2$ is continuously released as indicated by its tonic effects on platelet cAMP. In addition, WOS inhibited the contraction of aortic rings induced by PE, implying that WOS inhibits the release of intracellular Ca$^{2+}$ and/or blocks ROCC.

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**Conflict of Interests**

The authors declare that they have no conflict of interests.

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