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ORIGINAL ARTICLE

Autonomic Receptors and Nitric-Oxide Involvements in Mediating Vasorelaxation Effect Induced by *Syzygium polyanthum* Leaves Extract

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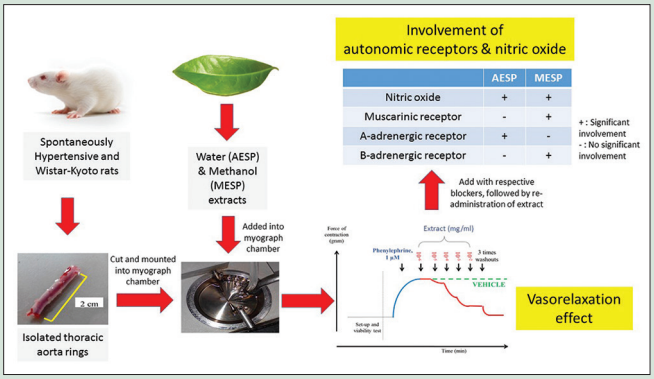
ABSTRACT

Context: *Syzygium polyanthum* (Wight) Walp leaves are traditionally used by Malays for treating hypertension. Our previous study showed that aqueous extract of *S. polyanthum* (AESP) and methanolic extract of *S. polyanthum (MESP)* extracts of *S. polyanthum* leaves significantly reduced blood pressure of normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). **Aims:** This study aimed to investigate their vasorelaxation potential and the possible involvement of autonomic receptors and nitric oxide in mediating their effect. **Settings and Design:** Both extracts will be tested on isolated thoracic aorta rings of WKY and SHR. The involvement of autonomic receptors and nitric oxide will be elucidated using respective blockers. **Materials and Methods:** Isolated thoracic aorta rings from WKY and SHR were mounted onto myograph chambers to measure changes in the aorta tension. Increasing concentrations of AESP and MESP, from 1 μg/ml to 10 mg/ml were added onto the myograph chambers. Blockers such as atropine (1 μM), phentolamine (1 μM), propranolol (1 μM), and Nω-nitro-l-arginine methyl ester (100 μM) were preincubated before addition of extracts to check for involvement of muscarinic, α- and β-adrenergic receptors (AR) as well as nitric oxide, respectively. **Statistical Analysis Used:** Two-way ANOVA, followed by *post hoc* Bonferroni test was used, where $P < 0.05$ (two-tailed) was considered statistically significant. **Results:** AESP and MESP caused significant vasorelaxations through nitric oxide pathway. The former was mediated through α-AR while the latter was mediated by β-adrenergic and muscarinic receptors. **Conclusion:** Vasorelaxation effect by AESP and MESP involved nitric oxide pathway which is possibly mediated by the autonomic receptors.

Key words: Antihypertensive, ethnomedicine, hypertension, nitric oxide, *Syzygium polyanthum*, vasorelaxation

SUMMARY

- This is the first study that reveals significant vasorelaxation effect induced by *Syzygium polyanthum* leaves extract. Vasorelaxation maybe one of the possible mechanisms for its ability to reduce blood pressure. This study also suggested that the vasorelaxation effect by this plant extract may involve nitric oxide pathway mediated by the autonomic receptors.



Abbreviations Used: AESP: Aqueous extract of *Syzygium polyanthum* leaves. MESP: Methanolic extract of *Syzygium polyanthum* leaves. SHR: spontaneously hypertensive rat, WKY: Wistar-Kyoto rat.

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DOI: 10.4103/pr.pr_69_17

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INTRODUCTION

Hypertension is an alarming disease condition. From 1990 to 2015, the prevalence of people with systolic blood pressure of 140 mmHg or higher has increased from 17.31% to 20.53%.[1] Ineffective treatment of hypertension may lead to increased risk of getting strokes, coronary thrombosis, and renal failure;[2] accordingly, this has led to an increased annual death rate related to hypertension. Despite abundance of conventional antihypertensive medicines, traditional remedy is still one of the best resources for discovering the further potential of new antihypertensive medicines with less of bothersome side effects.[3] This has driven more research to look into traditional pharmacopoeia from various parts of the world.[4-6] For instance, one of the Malay remedies for hypertension is by using *Syzygium polyanthum* (Wight) Walp fresh leaves or its decoction. Our previous study showed that intravenous administration of *S. polyanthum* leaves extracts caused a significant reduction in blood pressure of anesthetized normal and hypertensive rats.[7] The study also previously identified the possible involvement of autonomic receptors in mediating the reduction in blood pressure. In general, the autonomic nervous system, through the mediation of autonomic receptors from cholinergic and adrenergic nerve system regulates the vascular tone. Vascular tone affects the total peripheral resistance, thus contributes to the regulation of blood pressure. This study

Cite this article as: Ismail A, Wan Ahmad WA. Autonomic receptors and nitric-oxide involvements in mediating vasorelaxation effect induced by *Syzygium polyanthum* leaves extract. Phcog Res 2017;9:S9-14.

thus aimed to investigate the vasorelaxation potential of *S. polyanthum* leaves extracts on thoracic aorta rings, a commonly used subject in vasorelaxation study of normal and hypertensive rats.[3] In addition, this study also targeted to elucidate any possible involvement of nitric oxide (an endothelium-derived relaxing factor) and autonomic receptors in mediating the effect.

MATERIALS AND METHODS

Chemicals and reagents

Sodium pentobarbital (Dorminal 20%) was bought as an injectable solution (200 mg/ml, w/v) from Alfasan Woerden-Holland. Dimethyl sulfoxide (DMSO), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride dihydrate ($CaCl_2.2H_2O$), magnesium sulfate heptahydrate ($MgSO_4.7H_2O$), potassium dihydrogen phosphate (KH_2PO_4), D (+)-glucose, and sodium hydrogen carbonate ($NaHCO_3$) were purchased from Merck, Germany. Carbogen (a mixture of 95% O_2 and 5% CO_2) was purchased from Linde (M) Sdn. Bhd. Serotonin hydrochloride, phenylephrine hydrochloride, atropine sulfate, N_ω-nitro-l-arginine methyl ester (L-NAME), propranolol hydrochloride, and phentolamine hydrochloride were purchased from Sigma®, USA.

Plant materials

S. polyanthum leaves (0.52 kg) were collected from the District of Bachok, Kelantan, Malaysia. The plant was identified by a botanist, Dr. Richard Chung from the Forest Research Institute Malaysia (FRIM) as *S. polyanthum* (Wight) Walp var. *Polyanthum*. The herbal specimen (dried leaves) was deposited into FRIM herbarium (PID-171011-10). *S. polyanthum* leaves were extracted using protocols as reported in Ismail *et al.*[7] Basically, *S. polyanthum* leaves were washed with distilled water, dried at 50°C for 3 consecutive days, ground into powder, and the filtrate was sieved. Subsequently, the dry powdered sample was immersed in distilled water, heated at 80°C–90°C with continuous stirring for 30 min. The extract was filtered through Whatman No. 41 filter paper and then lyophilized. The lyophilized sample was designated as the aqueous extract of *S. polyanthum* leaves (AESP). The residue was then extracted using 95% methanol (v/v) in a Soxhlet apparatus for two continuous cycles. The extract was then concentrated through rotary evaporator and then dried at 50°C. The viscous extract was then designated as the methanolic extract of *S. polyanthum* (MESP). Both extracts were stored in-20°C freezer until use.

Preparation of extracts and drugs solutions

Kreb's Henseleit solution (KHS) was freshly prepared before experiment according to Jin *et al.*[8] For the preparation of KHS, NaCl (118 mM), KCl (4.7 mM), $MgSO_4.7H_2O$ (1.1 mM), KH_2PO_4 (1.2 mM), glucose (10 mM), and $NaHCO_3$ (25 mM) were dissolved in distilled water before use. $CaCl_2.2H_2O$ (1.5 mM) was finally added cautiously to the final solution with continuous stirring to eliminate the formation of salt, marked by whitish precipitates.

AESP and MESP were suspended in distilled water, but MESP was further added with DMSO so that the concentration in the final chamber would be 0.1% (v/v). DMSO, at a concentration of more than 0.1% (v/v) in a final chamber might affect the contractility of the blood vessel.[9] The extracts were then added to the 10 ml myograph chamber so that the final concentration of extracts would be 10 mg/ml, 1 mg/ml, 100 μg/ml, 10 μg/ml, and 1 μg/ml, respectively. All drugs were suspended in distilled water, while phentolamine hydrochloride and propranolol hydrochloride were suspended in distilled water plus 0.1% (v/v) DMSO. AESP and MESP solutions were then homogenized using a homogenizer (Ultra-Turrax® T25 Basic, Malaysia) at 24,000 per min for 3 min. All the extracts and the drugs solutions were vortexed using vortex machine (Erla) before use.

Animals

Male adult normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) (280–350 g) of 3–5 months old were supplied by Animal Research and Service Centre, Health Campus, Universiti Sains Malaysia. The research methodology was approved by the Animal Ethics Committee, Universiti Sains Malaysia with approval number, USM/Animal Ethics Approval/2012/(78) (374). These animals were housed in standard rat cages and were allowed to acclimatize for 7 days in a standard environmental condition (24°C with 60%–70% humidity) on a 12-h light-dark cycle. The animals were given standard rat pellets (Chipsi Classic Heimtierbett, Germany) and tap water *ad libitum*.

Preparation of rats' thoracic aortic rings

The rats were euthanized by a terminal dose of anesthesia using 100 mg/kg sodium pentobarbital through intraperitoneal injection. Rats were then laid in a supine position on the dissecting table. Immediately, the outer skin surrounding the chest area was removed, then the skin together with the rib cages were incised using a blunt-ended scissor. Using microforceps, the heart, the two lungs and the four lobes of liver were moved aside. The descending thoracic aorta was then identified. Two centimeters of the descending thoracic aorta was isolated and transferred into a petri dish-containing prewarmed and bubbled KHS solution with carbogen, a mixture of 95% O_2 and 5% CO_2. Then, the isolated descending thoracic aorta was cleaned from the adherent fat and connective tissues. Special care was taken to avoid endothelium damage. The two ends of the isolated thoracic aorta were carefully removed to minimize the shear-related damage upon isolation. The aorta was subsequently cut into 3 mm length rings. Four thoracic aorta rings were then mounted horizontally between the two mounting pins in four separated chambers of a multichannel myograph (Danish Myo Technology Model 620M, Denmark, USA) that was connected to PowerLab 8/30 data acquisition system (ADInstruments Ltd., USA). Each of the four chambers were loaded with 10 ml of KHS, warmed at 37°C using the integrated heating system, and were continuously aerated with carbogen. The responses (in grams) are displayed using LabChart Version 7.3.4 (ADInstruments Ltd., USA). One gram resting tension was set with reference to the previous studies as an optimal resting tension[10] for 30 min. Throughout the experiment, KHS was flushed by draining through the integrated automatic suction system. Immediately, another 10 ml of fresh KHS was transferred into each of the chambers using a 5 ml pipette. Each aorta rings were subjected to viability test by two repeated exposures to KCl (40 mM). Any rings that did not contract more than 50% from a normal response on addition of 40 mM KCl were excluded from the study.

Effects of *Syzygium polyanthum* leaves extracts on aorta rings from Wistar-Kyoto and spontaneously hypertensive rats

Thoracic aorta rings isolated from WKY and SHR rats were precontracted with phenylephrine (1 μM). Once the maximal effect of phenylephrine (1 μM) was achieved as indicated by the appearance of plateau region in the tracing, AESP or MESP solutions were cumulatively added to the chambers so that the final concentrations would be 1 μg/ml (10^{-6}), 10 μg/ml (10^{-5}), 100 μg/ml (10^{-4}), 1 mg/ml (10^{-3}), and 10 mg/ml (10^{-2}). In an experiment to investigate the involvement of α-receptors, thoracic aorta rings from WKY was precontracted with serotonin (10 μM). However, before the addition of serotonin, L-NAME (100 μM) was added to the chamber to avoid the fading of serotonin-induced contraction.[11] Once the maximal effect of serotonin (1 μM) in the presence of L-NAME (100 μM) was achieved as indicated by the appearance of a plateau region

in the tracing, then AESP or MESP solutions were cumulatively added to the chambers so that the final concentrations inside the chamber would be 1 μg/ml (10^{-6}), 10 μg/ml (10^{-5}), 100 μg/ml (10^{-4}), 300 μg/ml ($10^{-3.5}$), 1 mg/ml (10^{-3}), 3 mg/ml ($10^{-2.5}$), and 10 mg/ml (10^{-2}). In each experiment, subsequent additions of AESP or MESP solutions were made when the current concentration manifested its maximal effect and reached plateau.

From the force recordings (in grams), the vasorelaxation effects of AESP and MESP on aorta rings were determined using the following formula:

$$\frac{\text{Force}(\text{before}) - \text{Force}(\text{after})}{\text{Force}(\text{before})} \times 100 \quad = \text{Relaxation}(\%)$$

In this formula, the "Force (before)" is defined as the maximum force when phenylephrine or serotonin effect reaches plateau (in grams of tension); "Force (after)" is defined as the force after the addition of extracts (in grams of tension), and "Relaxation (%) is defined as relaxation to phenylephrine- or serotonin-induced contraction (in percentage). From the percentage relaxation, the cumulative concentration-response curve for AESP- and MESP-induced vasorelaxations on phenylephrine- and serotonin-induced contractions were constructed using GraphPad PRISM®.

Effects of autonomic receptors and nitric oxide production blockages on the vasorelaxation effects by aqueous extract of *Syzygium polyanthum* and methanolic extract of *Syzygium polyanthum*

To determine the involvement of α- and β-adrenergic, as well as muscarinic acetylcholine receptors and nitric oxide in mediating the effects of AESP and MESP on isolated thoracic aorta rings, phentolamine (1 μM), propranolol (1 μM), atropine (1 μM), and L-NAME (100 μM), respectively, were equilibrated in the chamber before the addition of precontractile agent and extracts.[6,12-14] Each antagonist was added to the chamber for 30 min before precontraction with 1 μM of phenylephrine. The period of 30 min was determined based on observation that the effects of KCl (40 mM) and phenylephrine (1 uM) apparently reached its maximal and became plateau within 30 min. Once the maximal effect of phenylephrine (1 uM) was achieved, as denoted by the appearance of plateau curve, then AESP or MESP solutions (1 μg/ml (10^{-6}), 10 μg/ml (10^{-5}), 100 μg/ml (10^{-4}), 1 mg/ml (10^{-3}), and 10 mg/ml (10^{-2}) were cumulatively added to the chambers. Each of the subsequent addition of the extracts was made when the current effect reached its plateau. In addition, all experiments utilized phenylephrine hydrochloride (1 μM) as a precontractile agent except for the experiment that investigated the involvement of α-adrenergic receptors (ARs). In the later, serotonin hydrochloride (10 μM) was used as a precontractile agent[11] in the presence of L-NAME (100 μM) to avoid the fading effect of serotonin alone; with and without blockage of α-ARs using phentolamine hydrochloride (1 μM).

Statistical analysis

Contractile responses were measured as mean increases in tension above the resting level (1 g) ± standard error of mean (SEM). Relaxant responses were expressed as mean reduction in tension (in percentage) from the induced precontraction ± standard error of the mean. Log concentration-response curves were fitted by sigmoidal curves through nonlinear regression equation using GraphPad PRISM v. 6.0.1 software for Windows (GraphPad, San Diego, CA, USA). Two-way repeated measures ANOVA test was used to determine the overall differences between AESP and MESP responses at different concentrations. A *post hoc* Bonferroni test was performed to compare the effects in between the concentrations. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Effects of *Syzygium polyanthum* leaves extracts on aorta rings from Wistar-Kyoto and spontaneously hypertensive rats

Vehicles for AESP and MESP did not cause any significant effect on isolated thoracic aorta rings of WKY [Figure 1a] and SHR [Figure 1b]. AESP significantly produced relaxation on isolated thoracic aorta rings of WKY at concentrations of 0.1 mg/ml (10^{-4}), 1 mg/ml (10^{-3}), and 10 mg/ml (10^{-2}) by 39.81 ± 2.99% ($P < 0.001$), 59.55 ± 7.60% ($P < 0.001$), and 72.58 ± 5.57% ($P < 0.001$), respectively, as compared to the vehicle [Figure 1a]. Similarly, MESP also significantly relaxed the thoracic aorta rings of WKY at concentrations of 0.1 mg/ml (10^{-4}), 1 mg/ml (10^{-3}), and 10 mg/ml (10^{-2}) by 48.53 ± 9.30% ($P < 0.001$), 76.69 ± 1.50% ($P < 0.001$), and 85.00 ± 4.96% ($P < 0.001$), as compared to the vehicle [Figure 1a].

In the meantime, test on isolated thoracic aorta rings of SHR showed that AESP produced significant relaxation at concentrations of

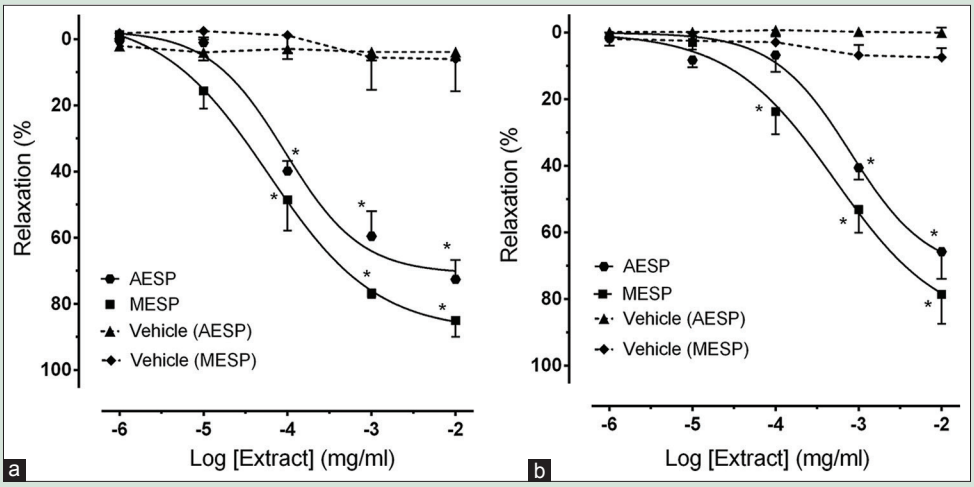

Figure 1: Concentration-response curve for extracts-induced relaxations in thoracic aorta rings ($n = 5$) of (a) Wistar-Kyoto and (b) spontaneously hypertensive rats which were precontracted using phenylephrine (1 μM). Relaxation (%): Percent relaxation (mean ± standard error of mean), AESP: Aqueous extract of *Syzygium polyanthum* leaves, MESP: Methanolic extract of *Syzygium polyanthum* leaves. *$P < 0.001$, extract versus vehicle

1 mg/ml (10^{-3}), and 10 mg/ml (10^{-2}) by 40.53 ± 3.66% ($P < 0.001$), and 65.73 ± 8.24% ($P < 0.001$), respectively, as compared to the vehicle [Figure 1b]. MESP also produced significant relaxation at concentrations of 1 mg/ml (10^{-3}) and 10 mg/ml (10^{-2}) by 53.08 ± 6.99% ($P < 0.001$) and 78.60 ± 8.86% ($P < 0.001$), as compared to the vehicle [Figure 1b]. There were significant differences between concentration-response curves for MESP-induced vasorelaxation in both isolated thoracic aorta rings of WKY and SHR at 0.1 mg/ml ($P < 0.05$) and at 1 mg/ml ($P < 0.05$).

Effects of autonomic receptors and nitric oxide production blockages on vasorelaxation effects by aqueous extract of *Syzygium polyanthum* and methanolic extract of *Syzygium polyanthum*

Muscarinic acetylcholine receptor blockage with atropine (1 μM) did not significantly affect AESP-induced vasorelaxation, but significantly reduced MESP-induced vasorelaxation at 0.1 mg/ml ($P < 0.05$) [Figure 2]. Phentolamine (1 μM) that blocks the α-ARs significantly reduced AESP-induced vasorelaxation at 1 mg/ml ($P < 0.001$) and at 3 mg/ml ($P < 0.001$), while similar concentration of phentolamine did not significantly affect MESP-induced vasorelaxation [Figure 3]. Beta-ARs blockage with propranolol (1 μM) did not significantly affect AESP-induced vasorelaxation, however, a similar concentration of propranolol significantly reduced MESP-induced vasorelaxation at 0.1 mg/ml ($P < 0.05$) [Figure 4]. In other experiments to determine involvement of nitric oxide, blockage with L-NAME (100 μM) significantly abolished AESP-induced vasorelaxation at 0.1 mg/ml ($P < 0.001$) and at 0.3 mg/ml ($P < 0.001$). The same concentration of L-NAME also significantly abolished MESP-induced vasorelaxation at 0.1 mg/ml ($P < 0.05$) [Figure 5].

DISCUSSION

This study showed that both aqueous and methanolic extracts of *S. polyanthum* leaves were able to cause significant vasorelaxation in both normal and hypertensive rats. This finding is in corroboration with our previous study, which indicated that both extracts were able to cause

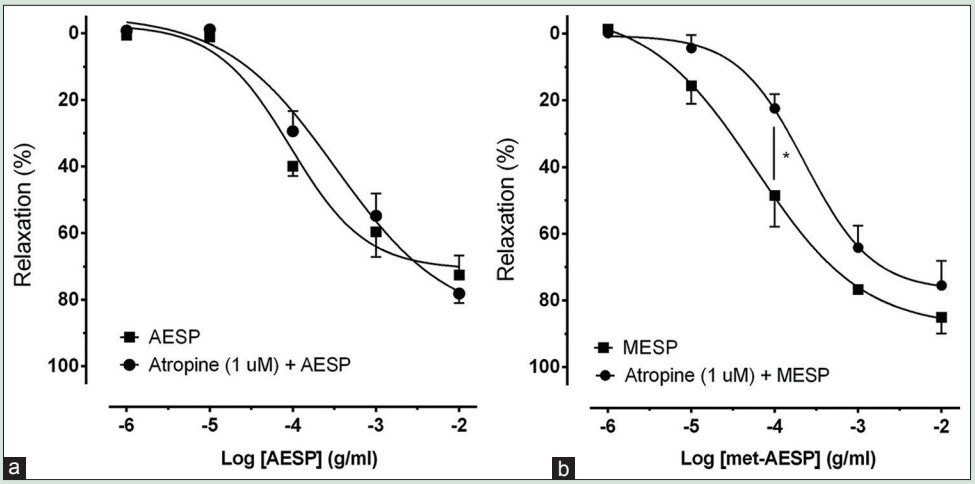

Figure 2: Concentration-response curves for the effect of muscarinic acetylcholine blockage on (a) Aqueous extract of *Syzygium polyanthum*- and (b) Methanolic extract of *Syzygium polyanthum*-induced vasorelaxation in isolated thoracic aorta rings of Wistar-Kyoto ($n = 5$). *$P < 0.05$, before versus after blockage

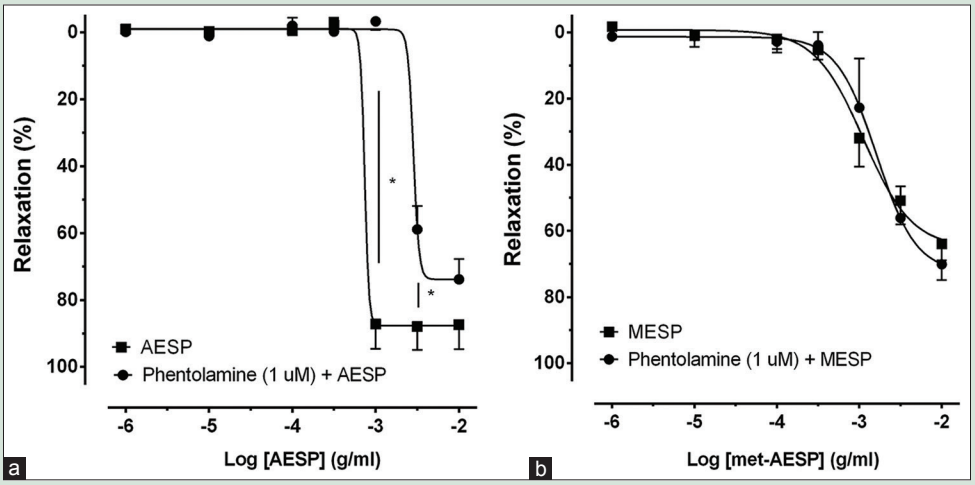

Figure 3: Concentration-response curves for the effect of α-adrenergic blockage on (a) Aqueous extract of *Syzygium polyanthum*-and (b) Methanolic extract of *Syzygium polyanthum*-induced vasorelaxations in isolated thoracic aorta rings of Wistar-Kyoto ($n = 5$). *$P < 0.001$, before versus after blockage

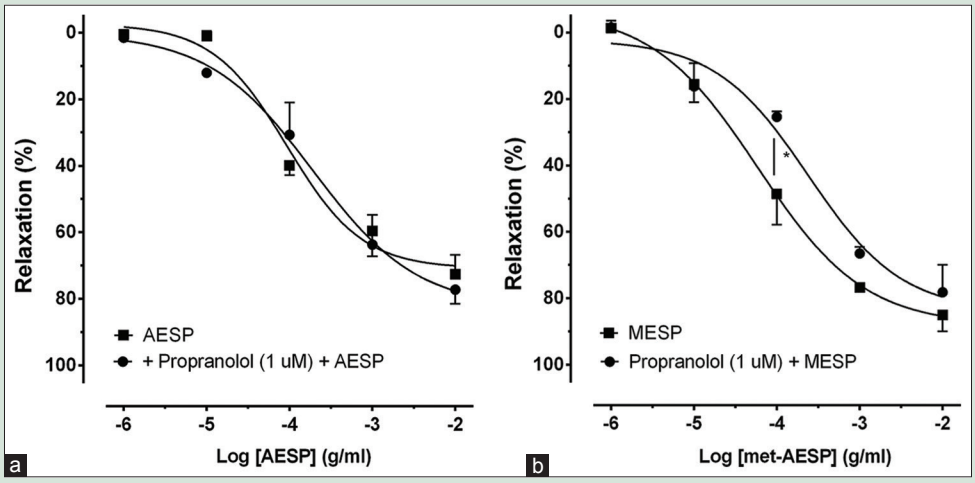

Figure 4: Concentration-response curves for the effects of β-adrenergic blockage on (a) Aqueous extract of *Syzygium polyanthum*-and (b) Methanolic extract of *Syzygium polyanthum*-induced vasorelaxations in isolated thoracic aorta rings of Wistar-Kyoto ($n = 5$). *$P < 0.05$, before versus after blockage

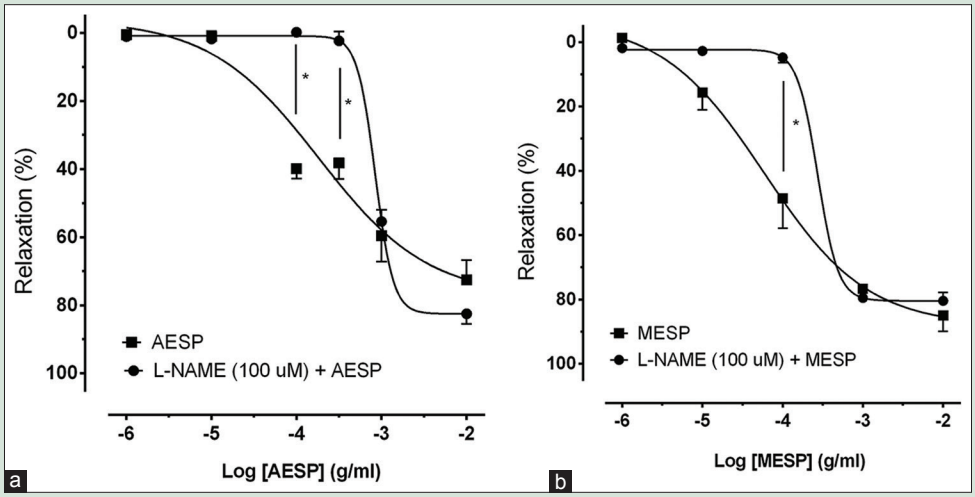

Figure 5: Concentration-response curves for the effects of endothelial nitric oxide synthase enzymes blockage on (a) Aqueous extract of *Syzygium polyanthum*- and (b) Methanolic extract of *Syzygium polyanthum*-induced vasorelaxations in isolated thoracic aorta rings of Wistar-Kyoto ($n = 5$). *$P < 0.001$, before versus after blockage

a reduction in blood pressure in both normal and hypertensive rats.[7] Reduction in blood pressure may be contributed by vasorelaxation, by means of reduced total peripheral resistance. In relation to this, a previous study on unripe grape juice has shown the potential relation between these two effects, through mediation of nitric oxide.[5]

It was evident from results of this study that both extracts produced more pronounced relaxation in normal than in hypertensive rats. A compromised vasorelaxation is a characteristic of vascular smooth muscle from animal models of hypertension. Indeed, an adult SHR with developed hypertension has an impaired endothelium-dependent relaxation, perhaps due to a decreased release of nitric oxide from dysfunctional endothelium.[15,16]

Our subsequent phase of the study investigated the involvement of autonomic receptors in mediating vasorelaxation effects by *S. polyanthum* leaves extracts. Vascular tone involves both controls from neural and humoral factors. This study focused on the neural factors which involves mediation of receptor-operated autonomic nervous system. Our experiment showed that blockage of muscarinic acetylcholine and β-ARs with their respective antagonists, atropine and propranolol, did not significantly affect AESP-induced vasorelaxation. However, pretreatment with phentolamine (a competitive blocker of α_1-and α_2-ARs) significantly affected AESP-induced vasorelaxation. This may suggest that there is a possible involvement of α-AR in mediating the vasorelaxation induced by AESP. Among the available subclasses of α-ARs, activation of α_{1D} and α_{2D}-ARs caused nitric oxide-mediated vasorelaxation.[17-20] Furthermore, the release of nitric oxide would attenuate vasoconstriction produced by activation of postjunctional vascular α_1-ARs.[17,21] The involvement of nitric oxide was investigated using L-NAME, a blocker of endothelial nitric oxide synthase (eNOS), an enzyme that is responsible for the production of nitric oxide. Indeed, this blockage significantly abolished AESP-induced vasorelaxations at lower concentrations. This observation has shown a prominent role of nitric oxide in mediating the effect of AESP.

Vasorelaxation induced by MESP was significantly affected by pretreatment with propranolol (a nonselective β-AR blocker) and atropine (a competitive muscarinic acetylcholine receptor blocker). MESP may partly acted through β-ARs. Among the available β-ARs

subtypes, β_2-and β_3 subtypes were previously reported to mediate nitric oxide-mediated vasorelaxation on thoracic aorta rings *in vitro*.[22-24] Since blockage with atropine also significantly affect MESP-induced vasorelaxation, it is also plausible to suggest a possible mediation by muscarinic receptors. M_1, M_2, and M_3 receptors mediated endothelium-derived vasorelaxation in various arterial preparations.[25] A significant abolished-effect on L-NAME pretreatment indicates for a prominent role of nitric oxide in mediating vasorelaxation effect by MESP.

CONCLUSION

Altogether, this study managed to discover the vasorelaxation properties of *S. polyanthum* leaves extracts. This indicates that vasorelaxation may partly contributes to the blood pressure-lowering ability of this plant. In addition, this study also suggested for an involvement of autonomic receptors, with the mediation of nitric oxide, an endothelium-derived relaxing factor. However, further in-depth study using more specific receptor blockers is recommended to confirm the subtype-specific autonomic receptors involved.

SUMMARY

This is the first study that reveals significant vasorelaxation effect induced by *Syzygium polyanthum* leaves extract. Vasorelaxation maybe one of the possible mechanisms for its ability to reduce blood pressure. This study also suggested that the vasorelaxation effect by this plant extract may involve nitric oxide pathway mediated by the autonomic receptors.

Financial support and sponsorship

This study was supported by USM-RU-PRGS Grant (1001/PPSK/8144010) from University Sains Malaysia and Research Initiative Grant Scheme (RIGS 15-039-0039) from International Islamic University Malaysia.

Conflicts of interest

There are no conflicts of interest.

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