Pharmaceutical Sciences Asia

Pharm Sci Asia 2024; 51(2), 145-154

DOI:10.29090/psa.2024.02.24.1292

Research Article

Evaluation of antioxidant, ACE inhibitory and vasorelaxant activities of *Murraya siamensis* leaf extract

Nungruthai Suphrom[1], Nitirut Nernpermpisooth[2,3], Porrnthanate seenak[2,3], Aticha Namwong[4], Sutida Yensukjai[4], Wachirawadee Malakul[4,5*]

[1] Department of Chemistry, Faculty of Science and Center of Excellence for Innovation in Chemistry, Naresuan University, Phitsanulok, Thailand
[2] Integrative Biomedical Research Unit (IBRU), Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand
[3] Department of Cardio-Thoracic Technology, Faculty of Allied Health Sciences, Naresuan University, Phitsanulok, Thailand
[4] Department of Physiology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand
[5] Centre of Excellence in Medical Biotechnology, Naresuan University, Phitsanulok, Thailand

ABSTRACT

Murraya siamensis Craib (Rutaceae) is a small shrub found in Asia, and its leaves have long been used for the treatment of various ailments. Nevertheless, the pharmacological activity of this plant is less documented. This study aimed to elucidate the antioxidant and angiotensin-converting enzyme (ACE) inhibitory properties of the ethanolic extract of *M. siamensis* leaves (MSE) and to explore the vasorelaxant effect of MSE and its underlying mechanisms. The antioxidant activity of MSE was evaluated by DPPH and ABTS radical scavenging assays, and its ACE-inhibitory activity was measured by the spectrophotometric method. Additionally, the vasorelaxant activity of MSE was determined on thoracic aortic segments isolated from Sprague Dawley rats. Results showed that MSE exhibited DPPH and ABTS scavenging activity with IC_{50} values of 710±10 and 488±20 µg/mL, respectively. MSE also showed ACE inhibitory activity with IC_{50} values of 1.01±0.02 mg/mL. Additionally, MSE (0.1-1 mg/mL) elicited a concentration-dependent relaxation in both endothelium-intact and -denuded aortic rings. The relaxant response to MSE was partially inhibited by pretreatment with tetraethylammonium or 4-aminopyridine but not in the presence of barium chloride or glibenclamide. Additionally, MSE (1 mg/mL) completely inhibited phenylephrine (PE)-induced transient contraction and abolished $CaCl_2$-induced contraction in the aortic rings pretreated with PE or 60 mM KCl. These results suggest that MSE has antioxidant and ACE inhibitory activities. Additionally, MSE exerts endothelium-independent vasorelaxant effects in the rat aorta through inhibiting intracellular Ca^{2+} release and extracellular Ca^{2+} influx via voltage-dependent and receptor-operated Ca^{2+} channels and opening K_{Ca} and K_V channels.

Keywords:
Murraya siamensis Craib, Vasorelaxation, ACE inhibition, Antioxidant activity

1. INTRODUCTION

High blood pressure or hypertension is the main cause of cardiovascular disease, which is one of the leading causes of death worldwide[1-4]. This disease is accompanied by damage to the blood vessels supplying various organs, which eventually results in life-threatening complications including stroke and kidney disease. Elevated blood pressure can be achieved by causing vasoconstriction, which is the crucial role of angiotensin converting enzyme (ACE)[1,5]. Thus, hypertension management focuses on pharmacological targets such as inhibiting ACE activity and dilation of blood arteries. Medically, several synthetic ACE inhibitors, such as captopril and enalapril, are commonly used in the treatment of hypertension; however, they have considerable side effects, including headaches, skin rashes, elevated blood potassium levels, dry coughs, and an abnormal taste[6]. Currently, the use of natural plant-based products for disease prevention, treatment, and healthcare is widely accepted because they are thought to be cheaper and have fewer adverse effects than synthetic drugs[7-8]. Therefore, searching for medicinal plants that inhibit ACE activity

***Corresponding author:**
*Wachirawadee Malakul Email: wachirawadeema@nu.ac.th

and have vasodilator properties is highly desirable because they have been proved effective in treating hypertension[9].

The species *Murraya siamensis* Craib, commonly known as 'Prong Fah' in Thai, is a small shrub found in tropical and subtropical areas of Thailand and Southeast Asia[10-13]. Fresh leaves of *M. siamensis* have a sweet and spicy taste and are very commonly used in traditional Thai cuisine[13]. The leaves and roots of *M. siamensis* have been used to treat various ailments such as flatulence, cough, sore throat, and asthma[10,13]. *M. siamensis* contains an abundance of bioactive compounds such as anethole, carbazole alkaloids, coumarin, and flavonoids[10-11,14], some of which have shown various biological properties including antihypertensive, antioxidant, hypolipidaemic and vasodilator properties[9,15-17]. There is evidence that plants containing flavonoids or coumarins have cardiovascular benefits, including lowering hypertension[18-19]. A pharmacological study reported the *in vitro* antioxidant activity of *M. siamensis* leaves[13]. In addition, coumarin, an active compound isolated from the leaves of *M. siamensis*, showed antitumor effects[10]. Other active compounds, the carbazole alkaloids, exhibited HIV-inhibitory effects[11] and neuroprotective effects against H_2O_2-induced cell damage[20].

Although some studies have reported the pharmacological activity of *M. siamensis* and its active compounds, such as coumarin and flavonoids, that have beneficial effects on cardiovascular health such as its antioxidant activity, there is no published pharmacological evidence of MSE's effectiveness in treating cardiovascular problems. Considering the growing usage of natural plants for preventing and treating hypertension, the present study was therefore undertaken to evaluate the ACE-inhibitory and antioxidant activity of the ethanolic extract of *M. siamensis* leaves. Furthermore, we investigated the impact of *M. siamensis* on the vascular tone of the rat thoracic aorta, and its possible mechanisms of vasorelaxant action.

2. MATERIALS AND METHODS

2.1. Preparation of *M. siamensis* Extract (MSE)

Fresh mature leaves of *M. siamensis* were collected from Pa Na Khok, Chiang Khan, Loei, Thailand in March 2021. The plant material was identified by Assist. Prof. Dr. Pranee Nangngam, Faculty of Science, Naresuan University. The voucher specimen (collection no. 05833) was deposited at the Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand. For the preparation of MSE, the fresh mature leaves of *M. siamensis* were chopped into small pieces, dried at 60°C, and ground into a fine powder. The *M. siamensis* leaf powder was macerated with 95% ethanol in a dry powder to solvent ratio of 1:20 w/v at room temperature, with occasional shaking (at least 5 days each time). The maceration process was repeated twice, and the solvent was removed under reduced pressure to produce a dark green, viscous crude ethanolic extract (MSE). The crude extract was kept at -20°C until used.

2.2. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

MSE was prepared at a concentration of 50 mg/mL in methanol and filtered using a 0.45 µm nylon filter. The sample was then injected into an Agilent 8890 gas chromatograph (GC)/7000D triple quadrupole GC/MS system (Agilent Technologies, Palo Alto, CA, USA). The condition parameters were set for the method described by Suphroma et al.[21] with some modifications. A fused silica capillary Hewlett Packard HP-5 (5% phenyl methyl siloxane) column (30 m × 0.25 mm × 0.25 µm film thickness) was used for the GC separation. High-purity helium with a constant flow rate of 1.0 mL/min was used as the carrier gas. The injector was adjusted to 250°C and operated in split mode with a 20:1 volume per volume in 1 µL split ratio. The oven temperature was maintained at 70°C for 3 min, then increased at a rate of 5°C/min to 280°C, and then maintained for 20 min. The transfer line heater was set at 280°C. The full mass scanning range was set as 50-550 amu. The identification of volatile components was accomplished by comparing the fragmentation patterns of their recorded mass spectra to those stored in the NIST17 MS spectral library. The relative amounts of each component in the sample were also determined based on the normalisation of peak areas as a percentage of the total detected volatile components.

2.3. Evaluation of total phenolic and flavonoid contents

The estimation of total phenolic content was done by using the Folin-Ciocalteu method[22]. Briefly, MSE (100 µL) was mixed with Folin-Ciocalteu reagent (0.5 mL), and subsequently 0.4 mL of a 7.5% (w/v) sodium carbonate solution (Na_2CO_3) was added to the reaction mixture. After incubation of the mixture at room temperature for 30 min, the absorbance was taken at 760 nm, and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE)/g of extract.

The total flavonoid content in MSE was measured according to the aluminium chloride method[22] Briefly, MSE (0.5 mL) was mixed with 0.2% aluminium chloride (0.5 mL) and 5% potassium acetate (3 mL). After incubation of the mixture at room temperature for 30 min, the absorbance was recorded at 430 nm, and the results were expressed as milligrams of quercetin equivalent (QE)/g of extract. All the determinations were performed in triplicates.

2.3. Evaluation of antioxidant capacity of MSE

The radical scavenging activity of MSE was deter-

mined using the diphenyl-picrylhydrazyl (DPPH) assay[23-24]. Briefly, MSE was added to 100 µL of DPPH radical solution (200 M) at concentrations of 0, 0.1, 0.5, 1, 5, and 10 mg/mL. After 30 min of incubation in the dark at room temperature, the absorbance at 517 nm was recorded.

The 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay[23-24] was performed to determine the antioxidant capacity of MSE. Briefly, different concentrations of MSE (0, 0.1, 0.5, 1, 5, and 10 mg/mL) were mixed with 7 mM ABTS solution (100 µL), then incubated in the dark at room temperature for 60 min, followed by the absorbance being measured at 734 nm. All the determinations were performed in triplicates.

Ascorbic acid was used as a reference standard, and the IC_{50} value, the concentration of MSE or ascorbic acid required to scavenge 50% of both DPPH or ABTS free radicals, was calculated as previously described[25].

2.4. Evaluation of *in vitro* ACE inhibitory activity

The ability of MSE to inhibit the activity of ACE *in vitro* was measured as previously described[26]. Briefly, 10 µl of MSE was added to 100 µl of 12.5 M hippuryl-L-histidyl-L -leucine (HHL) as substrate and incubated at 37°C for 5 min, followed by the addition of 150 µl of ACE enzyme (50 mU/mL). After 60 min of incubation at 37°C, the enzymatic reaction was stopped by adding 250 µl of 0.5 N HCl. The formation of hippuric acid by the reaction of the ACE with HHL was extracted with 1.5 mL of ethyl acetate, followed by centrifugation at 1200 × g for 5 min. The supernatant (0.5 mL) was collected and placed in boiling water to remove the ethyl acetate. The remaining hippuric acid was dissolved in 3 mL of 1 M NaCl and the absorbance was read at 228 nm. The ACE-inhibitory activity (%) was calculated as previously described[26]. Captopril was used as a reference standard. All the determinations were performed in replicates of six.

2.5. Experimental animals

Male Sprague-Dawley rats weighing 200-250 g were obtained from Nomura Siam International Co., Ltd (Bangkok, Thailand). The rats were housed in the animal room of the Centre for Animal Research, Naresuan University, Thailand at a constant temperature of 22±1°C, with a 12 h light and dark cycle and fed a standard diet and tap water *ad libitum* for 1 week. All animal experiments were approved by the Institutional Animal Care Committee of Naresuan University, Thailand (ethical approval number: NU-AE630920).

2.6. Vascular effect of MSE

Vascular reactivity was studied *in vitro* as previously described[27]. After rats were euthanised with an overdose of thiopental sodium (100 mg/kg), the thoracic aorta was isolated and was cut into 3-4 mm rings after removing its adventitial fats. In some experiments, the endothelial layer was taken off by carefully rubbing the intimal layer of the aortic segment using a tiny pair of forceps. The aortic rings were mounted under 1 g of resting tension in a standard 20 mL organ bath containing Krebs bicarbonate solution (118 mM NaCl, 25 mM $NaHCO_3$, 11.1 mM D-glucose, 4.7 mM KCl, 1.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$) which was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. Changes of vascular tension were measured with a force displacement transducer attached to a PowerLab device (AD Instruments, Hastings, UK). After 60 min of equilibration, aortic segments were maximally contracted with a high-potassium physiological salt solution (KPSS; isotonic replacement of NaCl with 122.7 mM KCl in Krebs bicarbonate solution). After washing with Krebs bicarbonate solution, the integrity of the vascular endothelium was assessed by the ability of acetylcholine (Ach; 10 µM) to induce relaxation of aortas submaximally pre-contracted with phenylephrine (PE; 0.01-3 µM). Endothelium-intact aortic rings were defined as those that exhibited at least 80% relaxation, whereas endothelial denudation was proven by less than 10% relaxation.

To determine the effect of MSE on vascular tension, both endothelium-intact and denuded rings were pre-contracted at 40-60% of their maximal contraction with PE. After steady contraction, cumulative concentrations of MSE (0.1, 0.25, 0.5, and 1 mg/mL) or vehicle (10% DMSO) were added to the organ chamber, and the vascular responses were recorded.

2.7. Effects of K^+channel inhibitors on MSE-induced vasorelaxation

Endothelium-denuded aortic rings were incubated with different types of K^+ channel inhibitors including the Ca^{2+}-activated K^+ (K_{Ca}) channel inhibitor tetraethylammonium (TEA; 5 mM), the voltage-dependent K^+ (K_V) channel inhibitor 4-aminopyridine (4-AP; 1 mM), the ATP-sensitive K^+ (K_{ATP}) channel inhibitor glibenclamide (10 µM), or the inward rectifier K^+ (K_{IR}) channel inhibitor barium chloride ($BaCl_2$; 1 mM) for 20 min, followed by their submaximal pre-contraction with PE (0.01-3 µM). Then, cumulative concentrations of MSE (0.01-1 mg/mL) were added to PE-precontracted rings and their vasorelaxant responses were recorded.

2.8. Effect of MSE on Ca^{2+}-induced vasoconstriction

The inhibitory effect of MSE on extracellular Ca^{2+} influx via receptor-operative Ca^{2+} channels (ROCCs) or voltage-dependent Ca^{2+} channels (VDCCs) in the endothelium-denuded rings was determined as previously described[28]. Briefly, in Ca^{2+}-free Krebs solution, the rings were pretreated with PE (1 µM) or KCl (60 mM) to produce

a steady contraction, and $CaCl_2$ (10 µM to 30 mM) was then added cumulatively to evoke a first contractile response (control). After washing with Ca^{2+}-free Krebs solution, $CaCl_2$-induced contraction of PE- or KCl-pretreated aortic rings was repeated in the presence of MSE (0.1, 0.5, and 1 mg/mL) or vehicle (10% DMSO).

The inhibitory effect of MSE on PE-induced intracellular Ca^{2+} release was investigated as previously described[28]. Briefly, the endothelium-denuded aortic rings were equilibrated in a Ca^{2+}-free Krebs solution containing 0.1 mM EDTA, and the first transient contraction of rings was then induced by PE (1 µM) (control). After washing with Krebs solution, the PE (1 µM)-induced transient contraction was repeated in the presence of MSE (0.1, 0.5, and 1 mg/mL) or vehicle (10% DMSO). The values were calculated as a percentage of the first contraction (control).

2.9. Statistical Analysis

Results are given as the mean±SEM (n=6-8), and n refers to the number of aortic rings from different animals. The vascular tension was expressed as a percentage of the maximum contraction response induced by PE. The concentration-response relationship to MSE was fitted to a sigmoidal curve using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA, USA) to calculate the sensitivity of MSE (EC_{50}). The statistical significance of differences was determined using a one-way analysis of variance (ANOVA), followed by Tukey HSD multiple comparison test (GraphPad Software Inc.). $P<0.05$ was taken as an indication of a statistically significant difference.

3. RESULTS

3.1. Volatile Compositions of MSE

The MSE was analysed by applying GC-MS and the total ion chromatogram is depicted in Figure 1. The volatile components were identified by comparing their MS spectra to the NIST17 MS spectral library. In total, 13 compounds were identified in the extract and these are shown in Table 1. The relative amount (%) of the compositions was determined using peak normalisation. The

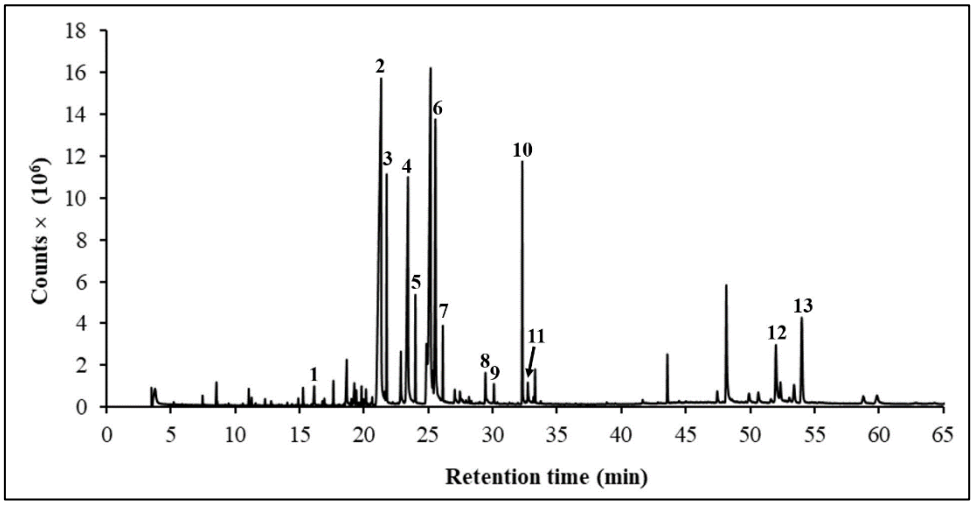

Figure 1. Total ion chromatograms of the ethanolic extract of *M. siamensis* leaves (MSE). The compounds assigned to the numbers are listed in Table 1.

Table 1. Volatile compounds of the ethanolic extract of *M. siamensis* leaves (MSE) identified using gas chromatography-mass spectrometry.

No.*	RT[1]	Identified compounds	Molecular formula	Classification	Relative amount (%)[2]
1	16.15	*n*-Decanoic acid	$C_{10}H_{20}O_2$	Fatty acid	0.50
2	21.34	Dodecanoic acid	$C_{12}H_{24}O_2$	Fatty acid	27.51
3	21.77	Ethyl dodecanoate	$C_{14}H_{28}O_2$	Fatty acid ester	3.80
4	23.43	Tridecanoic acid	$C_{13}H_{26}O_2$	Fatty acid	10.54
5	24.00	Ethyl tridecanoate	$C_{15}H_{30}O_2$	Fatty acid ester	1.84
6	25.55	Tetradecanoic acid	$C_{14}H_{28}O_2$	Fatty acid	8.62
7	26.13	Ethyl tetradecanoate	$C_{16}H_{32}O_2$	Fatty acid ester	1.32
8	29.44	Tetradecanoic acid	$C_{14}H_{28}O_2$	Fatty acid	0.55
9	30.10	Ethyl hexadecanoate	$C_{18}H_{36}O_2$	Fatty acid ester	0.35
10	32.30	Phytol	$C_{20}H_{40}O$	Diterpene	4.55
11	32.73	Acetoxyacetic acid, dodec-9-ynyl ester	$C_{16}H_{26}O_4$	Fatty acid ester	0.35
12	51.99	γ-Sitosterol	$C_{29}H_{50}O$	Sterol	2.46
13	54.00	Lupeol	$C_{30}H_{50}O$	Triterpene	0.87

*These numbers correspond to the numbers in Figure 1, [1]RT: Retention time (min); [2]Relative amount obtained by normalizing peak area.

present study showed the existence of many phytochemical constituents (fatty acids, fatty acid esters, diterpenes, sterols, and triterpenes). The majority of volatile chemicals detected were fatty acids (47.72%), followed by fatty acid esters (7.66%). MSE contained mostly dodecanoic acid (27.51%), tridecanoic acid (10.54%), and tetradecanoic acid (8.62%). Approximately 4.55% of the sample also included phytol, an acyclic hydrogenated diterpene alcohol. The other compounds were present in smaller percentages.

3.2. Total Phenolic and Flavonoid Content, and Antioxidant Activity of MSE

The total phenolic and flavonoid contents of MSE were 19.96±0.33 mg GAE/100 g and 5.42±0.71 mg QE/100 g, respectively. In this study, the capacity of MSE to scavenge DPPH and ABTS radicals was examined to assess its antioxidant activity. MSE (0.01 to 1 mg/mL) exhibited concentration-dependent radical scavenging activity by inhibiting DPPH and ABTS radicals with IC_{50} values of 710±10 and 488±20 µg/mL, respectively. The antioxidant activity of ascorbic acid, as a positive control, was shown to be more powerful than that of MSE, with IC_{50} values of 9.7±0.007 and 9.6±0.004 µg/mL for the DPPH and ABTS radical scavenging assays, respectively.

3.3. The ACE inhibitory activity of MSE

MSE showed inhibitory activity against ACE with IC_{50} values of 1.01±0.02 mg/mL. The extracts were less effective at inhibiting ACE than captopril, a synthetic ACE inhibitor which presented an IC_{50} value of 0.051±0.001 mg/mL.

3.4. Effect of MSE on endothelium-intact and -denuded aortic rings

The dual effect of MSE (0.01-1 mg/mL) was observed in aortic segments pre-contracted submaximally with both PE and KCl. The low concentrations of MSE (0.01-0.1 mg/mL) induced vasoconstriction of both endothelium-intact and -denuded aortic segments, and a concentration of MSE greater than 0.1 mg/mL reversed its contractile effect and induced a vasorelaxant effect.

As shown in Figure 2, MSE (0.1-1 mg/mL) generated a concentration-dependent vasorelaxation of endothelium-intact and denuded aortas precontracted with PE with a maximum relaxation (R_{max}) attained at the highest dose of 1 mg/mL. However, there was no statistically significant difference in the sensitivity and maximum response to MSE between the endothelium-intact rings (EC_{50}= 0.46±0.02 mg/mL and R_{max}=80±5%) and the endothelium-denuded rings (EC_{50}=0.51±0.09 mg/mL and R_{max}=89±6%). These results show that MSE's vasorelaxant activity primarily involves a direct impact on arterial smooth muscle.

3.5. Involvement of K^+ Channels in MSE-Induced Relaxation

Pre-incubation of endothelium-denuded aortic rings with 4-AP (1 mM) or TEA (5 mM) significantly reduced maximal relaxation of aortas in response to MSE (all $P<0.05$). However, the relaxant responses to MSE in the aortic rings were not affected by $BaCl_2$ or glibenclamide (Figure 3 and Table 2). These findings demonstrate that the vasorelaxant responses of rat aortic rings to MSE may be mediated by activation of K^+ efflux via K_{Ca} and K_V channels.

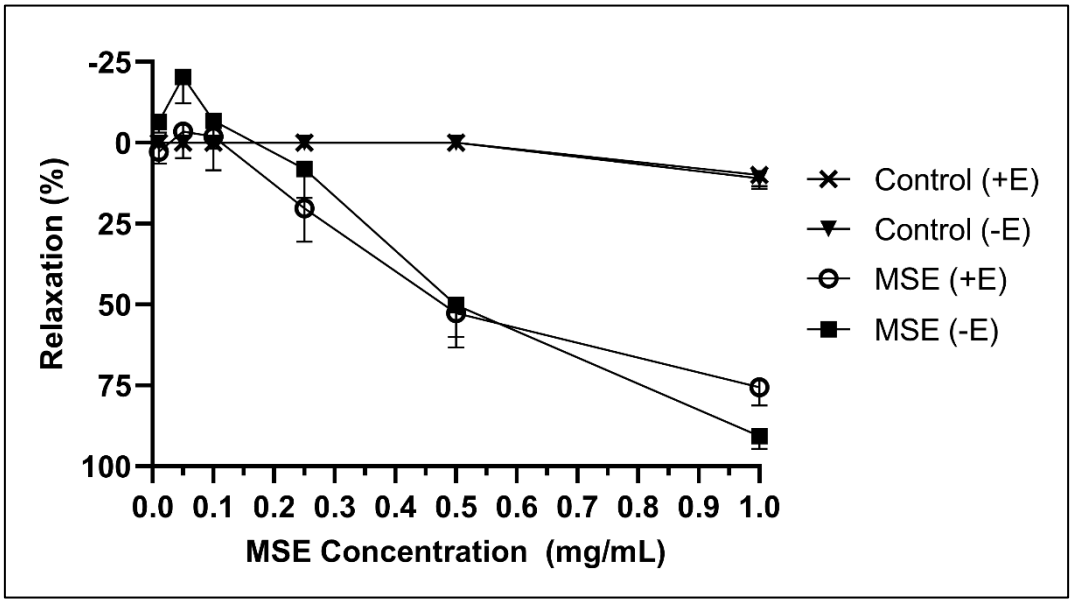

Figure 2. Cumulative concentration-response curves to MSE in endothelium-intact (E+) and endothelium-denuded (E-) aortic rings precontracted with PE. Data are presented as means±SEM of 6-8 experiments performed on preparations obtained from different animals.

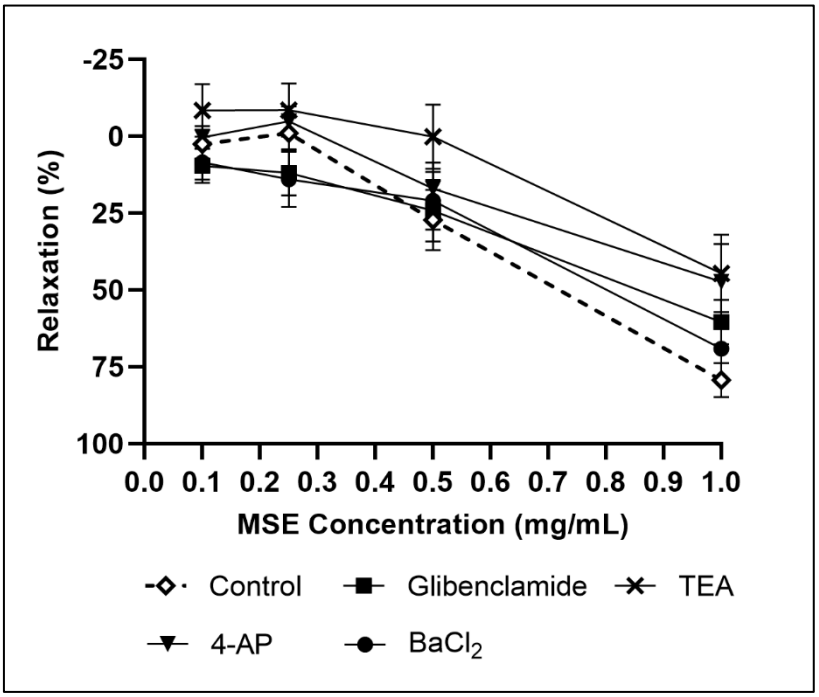

Figure 3. The vasorelaxant response induced by MSE in endothelium-denuded aortic rings in the absence (control) or presence of different K^+ channels inhibitors, including Ca^{2+} -activated K^+ (K_{Ca}) channels inhibitor tetraethylammonium (TEA, 5 mM), voltage dependent K^+ (K_V) channels inhibitor 4-aminopyridine (4-AP, 1 mM), ATP-sensitive K^+ (K_{Ca}) channels inhibitor (glibenclamide, 10 μM), and inward rectifier K^+ (K_{IR}) channels inhibitor barium chloride ($BaCl_2$, 1 mM). Data are expressed as the mean±SEM of 6-8 experiments performed on preparations obtained from different animals. The EC_{50} and R_{max} values determined from the data presented in these graphs are given in Table 2.

Table 2. Effects of different inhibitors on the EC_{50} and R_{max} values for MSE-induced relaxation in endothelium-denuded aortic rings.

	EC_{50} (mg/mL)	R_{max} (%)
Control	0.63 ± 0.11	79 ± 5
Glibenclamide	0.71 ± 0.09	61 ± 8
TEA	0.96 ± 0.09	45 ± 11*
4-AP	0.97 ± 0.09	47 ± 12*
$BaCl_2$	0.75 ± 0.12	70 ± 10

Data are the mean±SEM of six experiments. Relaxant responses to MSE were calculated as the percentage of contraction induced by PE or high concentration of KCl. EC_{50} represents concentration of MSE to produce 50% of the maximal relaxation. R_{max} represents the maximum vasorelaxation elicited by MSE at 1 mg/mL. $^*P<0.05$ compared to absence of antagonist (Control).

3.6. Involvement of extracellular Ca^{2+} Influx in MSE-Induced Relaxation

To determine whether MSE induces vasorelaxation through inhibiting VDCCs, aortic rings were incubated with 60 mM KCl to activate extracellular Ca^{2+} influx via the VDCCs. As shown in Figure 4a, in KCl-treated aortas, MSE at concentrations of 0.5 and 1 mg/mL significantly attenuated the maximum vasoconstriction induced by $CaCl_2$ to 38.6±10.6% and 7.5±3.4%, respectively (compared with the control group's value of 98.8±1.1%). To further investigate the involvement of the ROCCs in the relaxant response to MSE, the endothelium-denuded aortic rings were incubated with PE to activate extracellular Ca^{2+} influx via the ROCCs. In PE-pretreated aortic rings, MSE at concentrations of 0.5 and 1 mg/mL significantly inhibited $CaCl_2$-induced maximum contraction to 31.6±4.1% and 1.5±1.4%, respectively (compared with the control group's value of 90.2±4.8%). However, the low concentration of MSE (0.01 mg/mL) had no effect on contractile responses to cumulative concentrations of $CaCl_2$ in aortic rings pretreated with high KCl solution or PE (Figure 4a and Figure 4b). These results therefore suggest that MSE inhibits the influx of extracellular Ca^{2+} via both VDCCs and ROCCs in the aortas.

3.7. Inhibitory Effects of MSE on Intracellular Ca^{2+} Release

As shown in Figure 4c, MSE at concentrations of 0.5 and 1 mg/mL significantly attenuated PE-induced contraction, resulting in a significant decrease in maximal contraction to 10.9±1.1% and 3.8±2.1%, respectively (compared with the control group's value of 48.6±4.8%, all $P<0.01$). However, 0.01 mg/mL MSE had no significant effect on aortic contraction induced by PE. These results indicate that the vasorelaxant activity of MSE is mediated by a mechanism involving the inhibition of intracellular Ca^{2+} release.

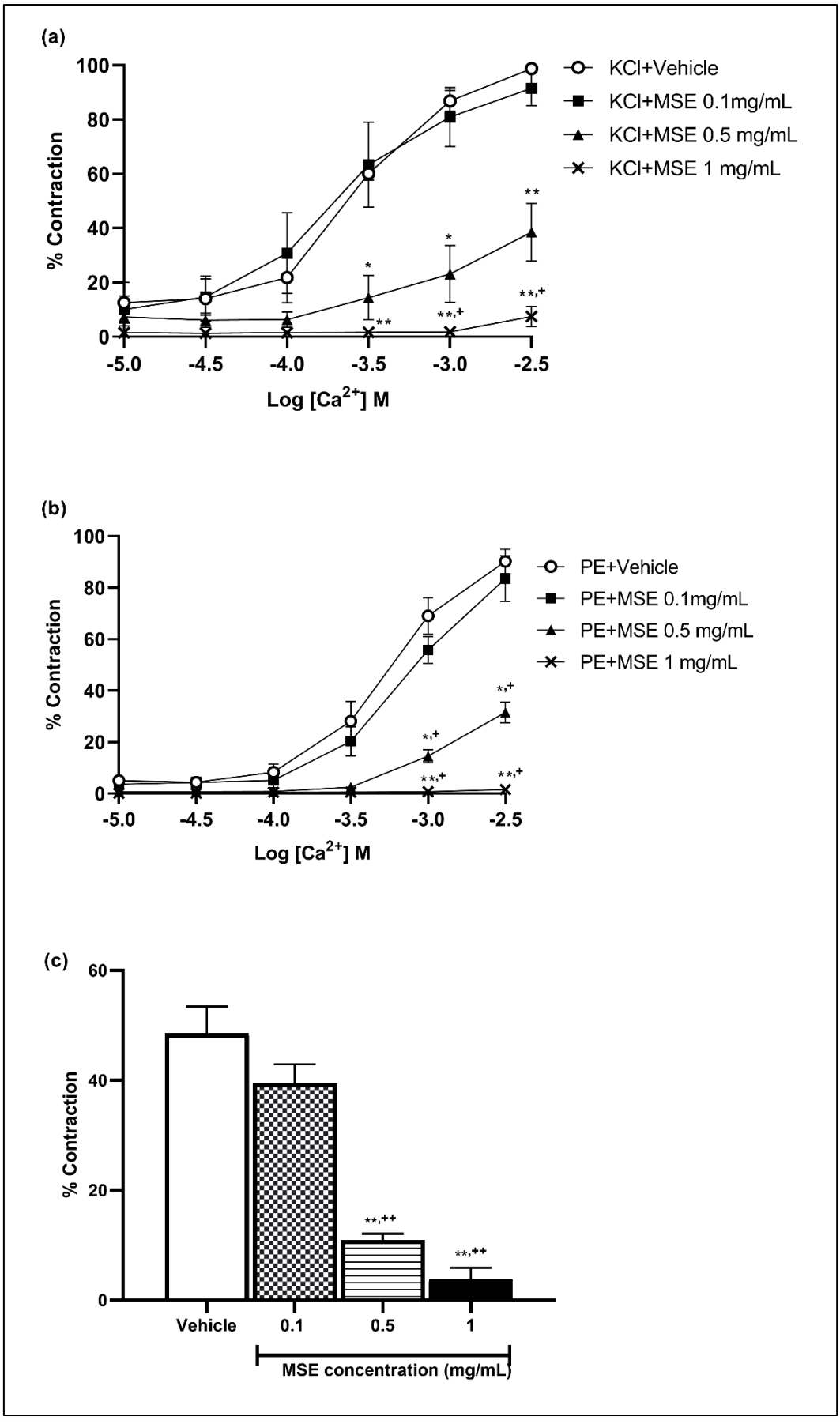

Figure 4. The inhibitory effect of MSE on $CaCl_2$ -induced contraction in aortic rings pretreated with 60 mM KCl (a) and PE (b), and PE-induced vasoconstriction (c). Data are expressed as the mean±SEM of 6-8 experiments performed on preparations obtained from different animals. $^{*}P<0.05$, $^{**}P<0.01$ compared to vehicle treatment; $^{+}P<0.05$, $^{++}P<0.01$ compared to MSE at concentration of 0.1 mg/mL treatment.

4. DISCUSSION

The present study demonstrated that MSE (0.01-1 mg/mL) has a dual effect on isolated aortic rings, both contraction and relaxation. MSE has a vasoconstriction effect at low concentrations (0.01 and 0.05 mg/mL) and exerts a vasorelaxant effect at higher concentrations (0.1, 0.25, 0.5, and 1 mg/mL). This study focused primarily on vasorelaxant mechanisms in the aortas. However, the mechanisms underlying the vasoconstriction of aortas triggered by MSE need further investigation.

It has been extensively documented that the endothelium plays a crucial role in controlling the contraction and relaxation of vascular smooth muscle cells (VSMCs) through the release of numerous substances derived from the endothelium, either relaxing or contracting, such as nitric oxide and endothelin-1[15,29]. This study is the first to report that MSE (0.1-1 mg/mL) induces concentration-dependent vasorelaxation in endothelium-intact rings. However, the relaxant response to MSE in aortic rings was largely unaffected by removing endothelial cells, indicating that its vasodilatory effects are mainly due to endothelium-independent mechanisms.

There are several K^+ channels in VSMCs, which play an important role in regulating VSM contraction by controlling cell membrane potential, such as the K_{Ca} channel, the K_V channel, the K_{ATP} channel, and the K_{IR} channel[30-31]. The activation of the K^+ channel in VSMCs causes membrane hyperpolarization and lowers the cytosolic Ca^{2+} content, which in turn causes vasorelaxation[30,32]. The results of this study showed that the vasorelaxant response to MSE was attenuated by pretreatment with TEA (K_{Ca} channel blocker) or 4-AP (K_V channel blocker) but was not affected by pre-incubation with $BaCl_2$ (K_{IR} channel blocker) or glibenclamide (K_{ATP} channel blocker). Therefore, the endothelium-independent vasorelaxation in response to MSE is partly associated with the opening of K_{Ca} and K_V channels.

The activity of Ca^{2+} channel is one of the main factors responsible for vascular tension. Depolarisation of VSMCs caused by high KCl solution leads to increased Ca^{2+} influx through the VDCCs, which results in vasoconstriction[29,31]. In contrast, the activation of α_1-adrenergic receptor by PE triggers extracellular Ca^{2+} influx through the ROCCs and releasing Ca^{2+} from the intracellular stores through the inositol 1,4,5 triphosphate (IP_3) pathway[31,33]. Our results showed that MSE was able to inhibit $CaCl_2$-induced contractions of the aortic rings subjected to PE or high KCl solution. These findings support the hypothesis that the vasorelaxant action of MSE is mediated by the blockade of both ROCCs and VDCCs. We further evaluate whether the vasorelaxant activity of MSE is involved in the inhibition of intracellular Ca^{2+} release. PE was used to activate the generation of IP_3 which results in the release of Ca^{2+} from the intracellular stores, followed by a transient contraction[31,33]. Our results indicated that PE-induced transient vasoconstriction was inhibited by pretreatment with MSE (0.5 and 1 mg/mL). These findings imply that another potential target of MSE-induced relaxation might be the suppression of Ca^{2+} release from intracellular reserves.

In this study, MSE demonstrated potent radical scavenging activity in the DPPH and ABTS assays. A significant body of evidence shows that the increase in reactive oxygen species (ROS) generation in response to pro-inflammatory factors such as angiotensin II induces nitric oxide inactivation, lipid peroxidation, and vascular remodeling. These all contribute to endothelial dysfunction, vasoconstriction, and vascular inflammation, which lead to the development and progression of hypertension[4,34]. There is growing evidence that the antioxidants found in natural products have a major role in maintaining human health and preventing diseases, including hypertension[35-36]. These antioxidant properties are generally attributed to the presence of polyphenolic compounds [24-25], but may also be associated with other compounds such as dodecanoic acid and tridecanoic acid[37-38]. Our study also found that MSE exhibited high ACE inhibitory activity. Inhibition of the enzyme ACE has been identified as an appropriate therapeutic approach for the regulation of blood pressure, as it plays a critical role in the production of angiotensin II, which causes vasoconstriction and oxidative stress, leading to elevated blood pressure[6]. Since MSE is able to reduce ACE activity, thereby inhibiting angiotensin II formation, which might partially lead to an activation of the K^+ channel and subsequent inhibition of Ca^{2+} influx through VDCCs[30,39-40].

This study's phytochemical screening of MSE revealed the presence of phenols and flavonoids, which have antioxidant and vasodilatory properties. The GC-MS analysis of MSE revealed that many fatty acids were present, mainly dodecanoic, tridecanoic, and tetradecanoic acids. Previous studies have shown that long-chain fatty acids (e.g. eicosapentaenoic acid)[41-42], and medium-chain fatty acids (e.g. dodecanoic acid)[42-43] have a vasodilatory effect. Another previous study demonstrated that dodecanoic acid, also known as lauric acid, lowers blood pressure and induces endothelium-independent vasorelaxation via inhibiting voltage-gated Ca^{2+} channels in the mesenteric artery of hypertensive rats[43]. Similarly, dodecanoic acid may also play a role in the vasorelaxant effects of MSE. Further research on these compounds is necessary to determine the precise mechanism behind the vasorelaxant effect of MSE.

5. CONCLUSION

This result is the first demonstration that MSE possesses ACE inhibition and antioxidant activities. The plant extracts was able to induce vasorelaxation mainly by directly affecting VSMCs. This effect was mediated by K_{Ca} and K_V channel activation, suppression of intracellular

Ca^{2+} release, and inhibition of Ca^{2+} influx via both ROCCs and VDCCs. Further investigation is needed to determine additional possible mechanisms underlying MSE-induced vasorelaxation and to isolate the compounds responsible for the observed pharmacological effects. At present, the pharmacological activity of these studied extracts provides basic scientific support for their ethnomedicinal use.

6. ACKNOWLEDGEMENT

We would like to thank Mr. Peter Barton at Naresuan University Language Centre (NULC), Naresuan University, Phitsanulok, Thailand for providing language editing assistance for our manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Funding

This research was supported by grants from the National Science, Research, and Innovation Fund, Thailand (NSRF; grant number: R2564B020).

Ethics approval

All animal experiments were approved by the Institutional Animal Care Committee of Naresuan University, Thailand (ethical approval number: NU-AE630920).

Article info:

Received November 5, 2023
Received in revised form February 10, 2024
Accepted February 17, 2024

REFERENCES

1. Kjeldsen S, Feldman RD, Lisheng L, Mourad JJ, Chiang CE, Zhang W, et al. Updated national and international hypertension guidelines: A review of current recommendations. Drugs. 2014; 74(17):2033-51.
2. Kim B, Kwon Y, Lee S, Lee K, Ham I, Choi HY. Vasorelaxant effects of *Angelica decursiva* root on isolated rat aortic rings. BMC Complement Altern Med. 2017;17(1):474.
3. Godskesen L, Abildgaard N, Kjeldsen J, Krag A. A rare cause of severe hepatomegaly with an improving outcome. BMJ Case Rep. 2014;2014:bcr2013203360.
4. Tain YL, Hsu CN. Oxidative stress-induced hypertension of developmental origins: Preventive aspects of antioxidant therapy. Antioxidants (Basel). 2022;11(3):511.
5. Lekoubou A, Bishu KG, Ovbiagele B. Nationwide impact of the 2017 American College of Cardiology/American Heart Association blood pressure guidelines on stroke survivors. J Am Heart Assoc. 2018;7(12):e008548.
6. Lee SY, Hur SJ. Antihypertensive peptides from animal products, marine organisms, and plants. Food Chem. 2017;228:506-17.
7. Rastogi S, Pandey MM, Rawat AK. Traditional herbs: A remedy for cardiovascular disorders. Phytomedicine. 2016;23(11):1082-9.
8. Auger C, Said A, Nguyen PN, Chabert P, Idris-Khodja N, Schini-Kerth VB. Potential of food and natural products to promote endothelial and vascular health. J Cardiovasc Pharmacol. 2016; 68(1):11-8.
9. Estrada-Soto S, González-Trujano ME, Rendón-Vallejo P, Arias-Durán L, Ávila-Villarreal G, Villalobos-Molina R. Antihypertensive and vasorelaxant mode of action of the ethanol-soluble extract from *Tagetes lucida* Cav. aerial parts and its main bioactive metabolites. J Ethnopharmacol. 2021;266:113399.
10. Ito C, Itoigawa M, Onoda S, Hosokawa A, Ruangrungsi N, Okuda T, et al. Chemical constituents of *Murraya siamensis*: Three coumarins and their anti-tumor promoting effect. Phytochemistry. 2005;66(5):567-72.
11. Meragelman KM, McKee TC, Boyd MR. Siamenol, a new carbazole alkaloid from *Murraya siamensis*. J Nat Prod. 2000;63 (3):427-8.
12. Esser HJ. Taxonomic notes on the Rutaceae of Thailand. Thai Forest Bull (Bot). 2021;49(1):27-31.
13. Yasin P, Maneepisamai M. Antioxidant activitity and chemical composition of *Murraya siamensis* Craib leaves. Agricultural Sci J. 2019;50(2):361-4.
14. Ruangrungsi N, Ariyaprayoon J, Lange GL, Organ MG. Three new carbazole alkaloids isolated from *Murraya siamensis*. J Nat Prod. 1990;53(4):946-52.
15. Li Z, Zhang F, Wang S, Xiao H, Wang J, Li X, et al. Endothelium-dependent vasorelaxant effects of praeruptorin a in isolated rat thoracic aorta. Bioengineered. 2022;13(4):10038-46.
16. Zhu JJ, Jiang JG. Pharmacological and nutritional effects of natural coumarins and their structure-activity relationships. Mol Nutr Food Res. 2018;62(14):e1701073.
17. Seo E, Kang P, Seol GH. Trans-anethole prevents hypertension induced by chronic exposure to both restraint stress and nicotine in rats. Biomed Pharmacother. 2018;102:249-53.
18. Shah SMA, Akram M, Riaz M, Munir N, Rasool G. Cardioprotective potential of plant-derived molecules: A scientific and medicinal approach. Dose Response. 2019;17(2):1559325819 852243.
19. Bachheti RK, Worku LA, Gonfa YH, Zebeaman M, Deepti, Pandey DP, et al. Prevention and treatment of cardiovascular diseases with plant phytochemicals: A review. Evid Based Complement Alternat Med. 2022;2022:5741198.
20. Zang YD, Li CJ, Song XY, Ma J, Yang JZ, Chen NH, et al. Total synthesis and neuroprotective effect of O-methylmurrayamine A and 7-methoxymurrayacine. J Asian Nat Prod Res. 2017;19(6): 623-9.
21. Suphroma N, Insumrong K, Ingkaninanb K, Boonphong S. Gas chromatography-mass spectrometry analysis and biological activities of hexane extract from *Boesenbergia xiphostachya* (Gagnep.) Loes. rhizome. Agr Nat Resour. 2019;53(5):472-8.
22. Prommaouan S, Nernpermpisooth N, Pengnet S, Paseephol T, Tunsophon S, Malakul W. Wax apple (*Syzygium samarangense*) fruit extract ameliorates endothelial dysfunction and liver damage in high cholesterol diet-fed rats. J Tradit Complement Med. 2022;12(6):584-93.
23. Carullo G, Ahmed A, Fusi F, Sciubba F, Di Cocco ME, Restuccia D, et al. Vasorelaxant effects induced by red wine and pomace extracts of Magliocco Dolce cv. Pharmaceuticals (Basel). 2020; 13(5):87.
24. Malakul W, Seenak P, Jumroon N, Arikit S, Kumphune S, Nernpermpisooth N. Novel coconut vinegar attenuates hepatic and vascular oxidative stress in rats fed a high-cholesterol diet. Front Nutr. 2022;9:835278.
25. Kefayati Z, Motamed SM, Shojaii A, Noori M, Ghods R. Antioxidant activity and phenolic and flavonoid contents of the extract and subfractions of *Euphorbia splendida* Mobayen. Pharmacognosy Res. 2017;9(4):362-5.
26. Sarmadi B, Ismail A, Hamid M. Antioxidant and angiotensin converting enzyme (ACE) inhibitory activities of cocoa (*Theobroma cacao* L.) autolysates. Food Res Int. 2011;44(1):290-6.
27. Tew WY, Tan CS, Asmawi MZ, Yam MF. Underlying mechanism of vasorelaxant effect exerted by 3,5,7,2',4'-pentahydroxyflavone in rats aortic ring. Eur J Pharmacol. 2020;880:173123.
28. Wang HP, Lu JF, Zhang GL, Li XY, Peng HY, Lu Y, et al. Endothelium-dependent and -independent vasorelaxant actions and mechanisms induced by total flavonoids of *Elsholtzia splendens*

in rat aortas. Environ Toxicol Pharmacol. 2014;38(2):453-9.
29. Haam CE, Byeon S, Choi S, Oh EY, Choi SK, Lee YH. Vasorelaxant effect of *Trachelospermi caulis* extract on rat mesenteric resistance arteries. Molecules. 2022;27(16):5300.
30. Jackson WF. Potassium channels in regulation of vascular smooth muscle contraction and growth. Adv Pharmacol. 2017;78:89-144.
31. Jackson WF. Ion channels and vascular tone. Hypertension. 2000;35(1 Pt 2):173-8.
32. Ch'ng YS, Loh YC, Tan CS, Ahmad M, Asmawi MZ, Wan Omar WM, et al. Vasorelaxant properties of *Vernonia amygdalina* ethanol extract and its possible mechanism. Pharm Biol. 2017; 55(1):2083-94.
33. Panthiya L, Pantan R, Tocharus J, Nakaew A, Suksamrarn A, Tocharus C. Endothelium-dependent and endothelium-independent vasorelaxant effects of tiliacorinine 12'-O-acetate and mechanisms on isolated rat aorta. Biomed Pharmacother. 2019;109:2090-9.
34. Amponsah-Offeh M, Diaba-Nuhoho P, Speier S, Morawietz H. Oxidative stress, antioxidants and hypertension. Antioxidants (Basel). 2023;12(2):281.
35. Obrenovich ME, Li Y, Parvathaneni K, Yendluri BB, Palacios HH, Leszek J, et al. Antioxidants in health, disease and aging. CNS Neurol Disord Drug Targets. 2011;10(2):192-207.
36. Ginter E, Simko V, Panakova V. Antioxidants in health and disease. Bratisl Lek Listy. 2014;115(10):603-6.
37. Sengupta A, Ghosh M, Bhattacharyya DK. *In vitro* antioxidant assay of medium chain fatty acid rich rice bran oil in comparison to native rice bran oil. J Food Sci Technol. 2015;52(8):5188-95.
38. Luo Y, Huang Y, Yuan X, Zhang L, Zhang X, Gao P. Evaluation of fatty acid composition and antioxidant activity of wild-growing mushrooms from Southwest China. Int J Med Mushrooms. 2017; 19(10):937-47.
39. Fan F, Sun CW, Maier KG, Williams JM, Pabbidi MR, Didion SP, et al. 20-Hydroxyeicosatetraenoic acid contributes to the inhibition of K+ channel activity and vasoconstrictor response to angiotensin II in rat renal microvessels. PLoS One. 2013;8(12):e82482.
40. Hayabuchi Y, Standen NB, Davies NW. Angiotensin II inhibits and alters kinetics of voltage-gated K(+) channels of rat arterial smooth muscle. Am J Physiol Heart Circ Physiol. 2001;281(6): H2480-9.
41. Engler MB, Engler MM, Ursell PC. Vasorelaxant properties of n-3 polyunsaturated fatty acids in aortas from spontaneously hypertensive and normotensive rats. J Cardiovasc Risk. 1994;1 (1):75-80.
42. Edirisinghe I, McCormick Hallam K, Kappagoda CT. Effect of fatty acids on endothelium-dependent relaxation in the rabbit aorta. Clin Sci (Lond). 2006;111(2):145-51.
43. Alves NF, de Queiroz TM, de Almeida Travassos R, Magnani M, de Andrade Braga V. Acute treatment with lauric acid reduces blood pressure and oxidative stress in spontaneously hypertensive rats. Basic Clin Pharmacol Toxicol. 2017;120(4):348-53.