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RESEARCH ARTICLE

Anti-hypertensive vasodilatory action of *Gynura procumbens* mediated by kaempferol 3-O-rutinoside

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Abstract

Introduction: *Gynura procumbens* (GP), otherwise known as longevity spinach or “Sambung Nyawa” in Malay, is an evergreen herb found in Africa and Southeast Asian countries (including Brunei) used traditionally to treat various diseases such as fever, diabetes and hypertension. We examined GP's vasodilatory action to determine its possible role via the cholinergic-mediated pathway.

Methods: GP leaves were prepared by filtration and evaporation to obtain the aqueous (AEGP) and methanol (MEGP) extracts followed by screening for phytochemical constituents. The total phenol, total flavonoid and flavonol contents were determined using the corresponding Folin-Ciocalteu, and aluminium colorimetric methods and the presence of kaempferol 3-O-rutinoside in the extracts was detected using HPLC analysis. Organ bath studies were conducted to determine the vasodilatory activity using intact and denuded isolated rat aortic rings by exposure to either increasing concentration of extracts (0.25, 0.5, 1.0, and 2.0 mg/mL) or 10 µg/mL kaempferol 3-O-rutinoside in the presence or absence of acetylcholine (ACh) after pre-contraction by noradrenaline (NA).

Results: MEGP contained more phytochemical constituents and higher content of total flavonoid and total flavonol but less phenolic content than AEGP. Furthermore, MEGP yielded a 20% elevated amount of kaempferol 3-O-rutinoside than AEGP. Both extracts significantly amplified ACh-endothelium dependent vasodilation and mediated relaxation at 1 mg/mL in endothelium-intact and endothelium-denuded aortic rings with MEGP as a more effective vasodilator than AEGP. Overall, these results imply the involvement of extracts in potentiating cholinergic pathway, which might be mediated by kaempferol, as shown by its vasorelaxation effects in endothelium-intact and -denuded aorta.

Conclusions: The present findings demonstrate that the vasodilatory activities of the two *Gynura procumbens* extracts, AEGP and MEGP, in thoracic aorta rings isolated from rats are potentially mediated via a cholinergic pathway through the action of a flavonoid particularly

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kaempferol 3-O-rutinoside.

Keywords

Gynura procumbens, Hypertension, Vasodilation, Cholinergic pathway, Kaempferol, Acetylcholine, Muscarinic receptors

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Introduction

Hypertension persists as high arterial pressure with systolic and diastolic blood pressure (BP) exceeding 140 and 90 mmHg, respectively¹ contributing to cardiovascular diseases (CVDs), which are the leading cause of death globally. An estimated 17.9 million people die annually from CVDs, representing 31% of all global deaths². More than 90% of hypertension is termed as essential or primary hypertension with unknown causes but influenced by environmental, genetic and behavioural factors^{3,4} including high salt intake, smoking, age, gender, obesity, diabetes, sedentary lifestyles, chronic alcohol consumption and family history of hypertension^{4,5}. The remaining 10% of the cases fall under secondary hypertension caused by potentially reversible biochemical or mechanical pathology associated with renal or adrenal diseases^{6,7}.

A continuous effort has been made to search for a safer and more effective method to control hypertension worldwide. Herbal medicines have been used since the beginning of human civilisation to control hypertension and other cardiovascular disorders including congestive heart failure, atherosclerosis, cerebral and venous insufficiency, arrhythmia and ischemic heart diseases⁸. Traditional herbs have become significant sources for drug research in pharmaceutical settings as alternatives to conventional synthetic drugs such as thiazide and captopril for hypertension⁹. *Gynura procumbens* (longevity spinach or “Sambung Nyawa”) is an evergreen herb that is used traditionally in Africa and Southeast Asia including Brunei, Indonesia, Thailand and Malaysia to treat various diseases such as hypertension, rashes, kidney disease, fever and diabetes^{10,11}. Belonging to the Asteraceae family, this plant is small in size (around 1-3 m in height) with oval-shaped green leaves, fleshy stems and purple tint¹², and can thrive in partial, shaded or full sun as long as the soil is moist. The leaves of this plant are traditionally consumed as salad, juice or tea for health benefits.

Various studies have demonstrated the antihypertensive activity of GP both *in-vitro* and *in-vivo*^{8–10,13–16}, acting to lower the blood pressure by stimulating vasodilation besides heart stabilisation and diuretic effects. However, its mechanism of inducing vasodilation is not well understood. Activation of the parasympathetic division of autonomic nervous system induces relaxation of the blood vessel¹⁷ via the cholinergic pathway by releasing acetylcholine (ACh) from the nerve terminal in blood vessel walls, which in turn stimulates nitric oxide (NO) production that triggers dilation of vascular smooth muscle. The identity of GP's active ingredient for vasodilation is likely to be the flavonoids, which may be responsible for its antihypertensive effect^{10,14,18,19}. Kaempferol is a flavonoid that is present in the sub-fraction of the ethanolic extract of GP^{20,21}, and kaempferol 3-O-rutinoside is one of the various types of kaempferol that has been identified in GP¹⁴. In this present study, we investigated GP's vasodilatory action and, then, determined its possible activation of the cholinergic pathway mediated by kaempferol 3-O-rutinoside.

Methods

Chemicals and Reagents

All chemicals used for methanol extraction, phytochemical content determination including total phenolic, flavonoid, flavonol and high liquid performance liquid chromatography (HPLC) were obtained from Merck, Germany except for Dragendorff's reagent, mercuric chloride, potassium iodide, iodine, quercetin and kaempferol 3-O-rutinoside, which were purchased from Sigma Aldrich, USA. All reagents for organ bath studies including those to prepare Kreb's Henseleit solution (Kreb's solution), sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), glucose, sodium hydrogen carbonate (NaHCO_3) and calcium chloride (CaCl_2) were purchased either from Sigma Aldrich or Merck.

Preparation of solution

Kreb's solution was prepared by dissolving compounds in distilled water and adding calcium chloride last with continuous stirring to eliminate the formation of a salt precipitate. For the organ bath study, drugs used were dissolved following the manufacturer's instructions and GP extracts were dissolved in Kreb's solution. For HPLC experiment, kaempferol 3-O-rutinoside was dissolved in methanol.

Plant collection and verification

GP was purchased from Rimba Horticulture Centre, Brunei Darussalam. Leaf samples were then verified by Dr Johan Willem Frederick Slik (Associate Professor in Phylodiversity, Faculty of Science, Universiti Brunei Darussalam). The herbal specimen (dried leaves) was deposited into Universiti Brunei Darussalam Herbarium (UBDH) with a sample number 17H8182.

Drying of *G. procumbens* leaves and powdered preparation

Fresh leaves of GP were cleaned, weighed, and oven-dried at 60°C for three days. The dried leaves were then crushed using a blender (Waring 800s, USA) to produce fine powder and filter-sieved in dry form using a mechanical sieve (0.3 mm mesh hole size).

Preparation of aqueous (AEGP) and methanol extracts of *G. procumbens* (MEGP)

Aqueous extract of *Gynura procumbens* (AEGP) was prepared as described previously²². Briefly, the powdered leaves were macerated twice in distilled water (1:10 ratio of solute to solvent) and kept at 60°C water bath for three days. After filtration, the filtrate was centrifuged at 3000 rpm for 20 min, and the supernatant was recovered, and frozen at -80°C overnight. The sample was then lyophilised by freeze-drying for three days to yield a brown powder of AEGP²³ that was stored at 4°C until further use.

MEGP was prepared as described previously²⁴ with slight modifications, using ultrasonic extraction (UE) with 95% methanol in 300 W ultrasonic bath. Briefly, the powdered leaves were

placed in a beaker and added with methanol at a 1:20 (solute: solvent) ratio before subjected with ultrasound at 25°C for 30 min. After extraction, the sample was filtered using filter paper, and the powder residues were added again with methanol of 1:20 (solute: solvent) ratio in another cycle of ultrasound. The filtrate obtained was evaporated with a rotary evaporator (Yamato RE301, Japan) at 63°C, and left to dry at 60°C in a drying cabinet for five days yielding a dark greenish-brown sticky solid MEGP that was stored at 4°C until further use. The percentage of yield was calculated using the following equation²⁵:

$$\% \text{ Yield} = \frac{\text{Mass of plant crude extract}}{\text{Mass of powder plant in thimble}} \times 100\%$$

Phytochemical screening

The extracts were tested using the standard procedures for phytochemical constituents, including alkaloids, glycosides²⁶, flavonoids, saponins, steroids, triterpenoids²⁷ and tannins²⁸. For stock extract solution preparation, AEGP and MEGP (1 g each) were completely dissolved in 100 mL of water and methanol, respectively. The stock solutions obtained were used for phytochemical screening whenever necessary as some of the methods required the solid form of the extracts. Qualitative results were expressed as (+) for the presence and (-) for the absence of phytochemicals. Each test was done by visual observation of colour change or formation of a precipitate after the addition of specific reagents. Hence, the experiments were only carried out once.

Test for alkaloids

Around 15 mg of each extract was separately stirred in 6 mL of 1% HCl at room temperature water bath for 5 min and then filtered using Whatman filter paper (grade 42, 55 mm). The filtrates were divided into two equal parts and used for Dragendorff's and Mayer's tests. The orange-red precipitate formation indicated the presence of alkaloids using Dragendorff's test upon addition of 1 mL of Dragendorff's reagent (potassium bismuth iodide solution) to the first extract filtrate. Using Mayer's test, the presence of alkaloids was indicated by the cream-coloured precipitate or turbid solution formation upon addition of 1 mL of Mayer's reagent (potassium mercuric iodide solution) to the second filtrate.

Test for flavonoids

The presence of flavonoids was detected by the formation of an intense yellow colour solution that turned into colourless upon addition of few drops of 1 M HCl to a mixture of 1 mL of each extract and 0.2 mL of 1 M NaOH.

Test for glycosides

Using Borntrager's test, the presence of anthraquinone glycosides was detected by the formation of rose-pink to red colour ammoniacal layer upon addition of diluted (10%) ammonia to the lower layer of chloroform that was recovered from boiling and filtering a mixture of 1 mL of each extract, 1 mL of 5% sulphuric acid (H₂SO₄) and 2 mL chloroform.

Test for saponins

The presence of saponins was detected by the formation of a topmost foamy layer upon thoroughly mixing by hand a dilution of 1 mL of each extract in 20 mL of distilled water for 15 min.

Test for steroids

The presence of steroids was detected by the colour change of the upper layer into red and sulphuric acid (H₂SO₄) layer, and into fluorescent yellow upon addition of 10 mL of chloroform and an equal volume of concentrated H₂SO₄ into 1 mg of each extract.

Test for tannins

The presence of tannins was detected by the development of precipitate upon addition of 1% potassium dichromate solution to an equal volume of each extract.

Test for triterpenoids

The formation of reddish-violet colour detected the presence of triterpenoids upon addition of 2 mL of chloroform, 1 mL of acetic anhydride and 1 mL of concentrated H₂SO₄ to 5 mg of each dry extract.

Determination of total phenolic, total flavonoids and total flavonol contents

Total phenolic content. The total phenolic content of each plant extract was determined using Folin–Ciocalteu assay^{10,22,26}. Briefly, 20 µL of extracts, 100 µL of 2 N Folin–Ciocalteu phenol reagent and 1.58 mL of distilled water were mixed thoroughly. After 8 min, 300 µL of Na₂CO₃ was added into the mixture and allowed to react at room temperature for two hours before measuring the absorbance at 765 nm using a spectrophotometer. A standard calibration curve of gallic acid (0–4 mg/mL) was prepared, and results were expressed as mg of gallic acid equivalent (GAE) per g of extract. Experiments were replicated four times.

Total flavonoid content. Total flavonoid content of each extract was determined using aluminium colourimetric method^{10,22,26} using quercetin (0–200 µg/mL) as a standard. Briefly, 0.5 mL of extract and 0.5 mL of the standard were placed in separate test tubes with 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, 1.5 mL of 80% methanol and 2.8 mL of distilled water, and mixed thoroughly. A blank was prepared in the same manner where 0.5 mL of distilled water was used instead of the sample or standard, and the aluminium chloride replaced by distilled water. All tubes were incubated at room temperature for 30 min, and the absorbance was measured at 415 nm using the spectrophotometer. Experiments were carried out four times. Results were expressed as mg of quercetin equivalent (QE) per gram of extract.

Total flavonol content. Total flavonol content of each extract was analysed using aluminium chloride colourimetric method as described previously²⁶. Briefly, quercetin was used to make

a standard calibration curve ranging from 0 to 100 µg/mL. The test tube containing 1 mL of extract or 1 mL of standard solution was added with 1 mL of 2% aluminium chloride and 3 mL of 5% sodium acetate and then mixed well. The mixture was then centrifuged at 3000 rpm for 20 min to get a clear solution. The absorbance of the standard and sample was measured at 440 nm using the spectrophotometer. Experiments were replicated four times. Results were expressed as mg of quercetin equivalent (QE) per gram of extract.

High-performance liquid chromatography (HPLC) analysis of kaempferol-3-O-rutinoside

HPLC analysis was performed as previously described²⁹ using Agilent 1200 series HPLC system (Agilent LabX, USA) equipped with an autosampler and coupled to a variable UV wavelength detector. Liquid chromatography was performed on a reversed-phase C18 column (internal diameter 4.6 mm, height 250 mm, and particle size 5 µm). The mobile phase consisted of two solvents ([A] 5% CH₃COOH, acetic acid and [B] CH₃CN, acetonitrile) using the following elution program: 0–10 min 70% A isocratic, 10–20 min gradient to 40% A and then 20–30 min 40% A isocratic again with a flow rate of 1.0 mL/min at 30°C to detect peaks at 367 nm. Standard calibration was done using kaempferol 3-O-rutinoside at a concentration ranging from 0 µg/mL to 100 µg/mL. After the calibration curve was generated, both extracts with or without spiking with kaempferol 3-O-rutinoside (100 µg/mL) were run using the same elution program. Experiments were carried out in triplicates.

Animals

A total of 25 adult male Sprague Dawley (SD) rats at age of 16–20 weeks, weighing from 250 to 300 g, were obtained from Universiti Brunei Darussalam animal house. The animals were housed in standard environmental conditions (24°C, 60–70% humidity) on a 12 hour-light-dark cycle feeding. The animals were given standard rat pellets and tap water *ad libitum*.

Statement on ethical issues

The study was conducted following the ethical guidelines of the Laboratory Animal Care (NIH publication #85-23, revised in 1985) and approved by the University Research Ethics Committee (UREC) of PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam.

Vessel preparation and setting-up of the organ bath

After sacrificing the rats by cervical dislocation, their descending thoracic aorta was removed and placed in a petri dish on ice containing Krebs's buffer (118.2 mM of NaCl, 4.7 mM of KCl, 2.5 mM of CaCl₂, 25 mM of NaHCO₃, 1.2 mM of MgSO₄, 1.2 mM of KH₂PO₄, and 11.7 mM of glucose). The rat aorta was dissected, cleared of fats including connective tissues, and cut into rings of 3 mm long. The denuded endothelium was prepared by inserting fine forceps into the lumen of the aorta with gentle rotation. The aortic rings were mounted using two triangular-shaped platinum wire holders in the vessel lumen and suspended horizontally in an organ bath with 10 mL of Krebs's buffer, thermoregulated at 37°C, and bubbled with carbogen to a resting tension of 1.0 g (Figure 1A). One of the holders was fixed to the bottom of the chambers, and the other was connected to a force-displacement transducer (Grass®

Force Displacement Transducer FT03) for measuring isometric tension. The rings were equilibrated at 1.0 g resting tension for 20 min, with bathing solution replacement every 10 min, and tension readjustment to 1.0 g if necessary. The aortic rings were tested for viability using 40 mM KCl before conducting experiments (Figure 1B). Isometric contraction and relaxation were recorded using a Force Displacement Transducer. The transducer signals were displayed, stored and analysed on a computer using AD Instruments model 4/25T with Power Lab Software version 7. The responses (in grams) were recorded using LabChart Reader 7.

Experimental protocols

The following experiments were conducted using both endothelium-intact and endothelium-denuded aorta, except for the protocol on the addition of noradrenaline (NA) with the extracts or kaempferol, whose effects were previously determined and ruled out as NA-independent regardless of the tissue preparation.

Effects of AEGP and MEGP on aortic rings pre-contracted with NA

The aortic rings were pre-contracted by adding 50 nM of NA to induce transient and stable contraction for 60 min to generate the first response curve. Then, the aortic rings were washed with Krebs's buffer three times, allowing the baseline to stabilise for 10 min before pre-contracted again with 50 nM of NA. After the tonic response or contraction became stable for 20 min, increasing concentrations of AEGP or MEGP (0.25, 0.5, 1 and 2 mg/mL) were added cumulatively at 10 min interval to obtain the second response curve.

Effect of AEGP and MEGP and their potential influence on the cholinergic-mediated pathway

The aortic rings were pre-contracted with 50 nM of NA for 10 min, followed by addition of 100 µM of ACh for 50 min to obtain the first response curve. Then, the aortic rings were washed with Krebs's buffer three times, allowing the baseline signal to stabilise for 10 min. The aortic rings were pre-contracted again with 50 nM of NA for 10 min followed by addition of 100 µM of ACh for another 10 min before adding AEGP or MEGP cumulatively in increasing concentrations (0.25, 0.5, 1 and 2 mg/mL) every 10 min to obtain the second response curve.

Effect of kaempferol 3-O-rutinoside on NA-induced contraction

The aortic rings were pre-contracted with 50 nM of NA for 30 min to obtain the first response curve. Then, the aortic rings were washed with Krebs's buffer three times, allowing the basal tension signal to stabilise for 10 min. The rings were pre-contracted again with 50 nM of NA for 10 min, followed by addition of 10 µg/mL kaempferol 3-O-rutinoside for 20 min to generate the second response curve. This experiment was to rule out the role of kaempferol in enhancing the ACh-mediated vasodilation.

Effect of kaempferol 3-O-rutinoside on ACh-mediated vasodilation

The aortic rings were pre-contracted with 50 nM of NA for 10 min, followed by addition of 100 µM of ACh for 20 min to obtain the first response curve. Then, the aortic rings

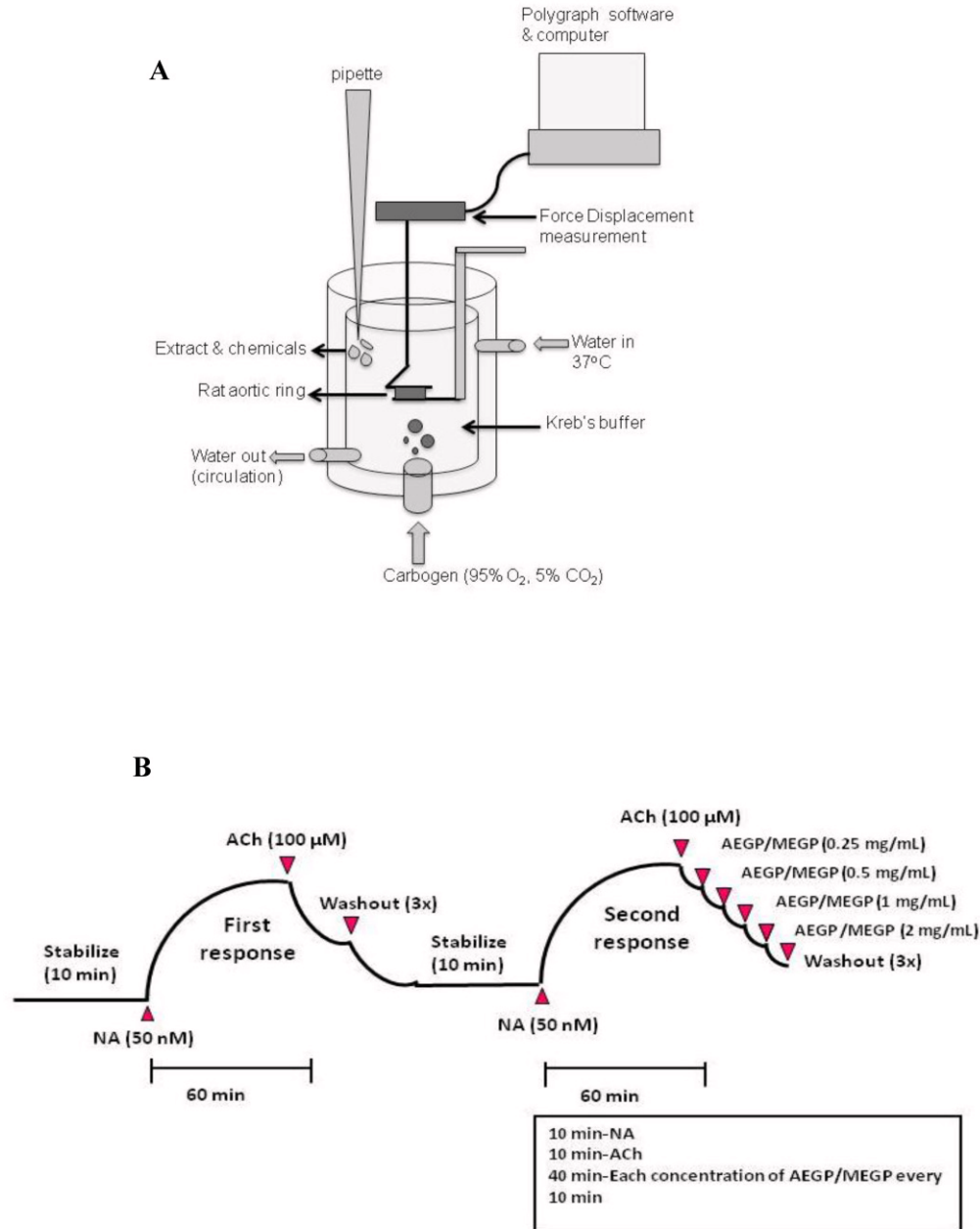


Figure 1. Isolated organ bath system. (A) Schematic set-up of the organ bath. The aortic rings were mounted using two-triangle-shaped platinum wire holders, suspended horizontally in an organ bath containing 10 mL of the above solution, thermoregulated at 37°C, and bubbled with carbogen with a resting tension of 1 g. Isometric contraction and relaxation were recorded using Force Displacement Transducer. Transducer signals were displayed and stored on a computer. (B) A summarised protocol of one of the organ bath experiments. Before each experiment, the aortic rings were stimulated with 40 mM KCl to produce a contraction, which represents a reference response, ensuring the viability of the rings just before the start of the experiment. Then the buffer solution was replaced for three times, and baseline was stabilised before adding 50 nM of NA for 10 min to induce stable contractions, and 100 μM of ACh was added for 50 min afterwards to produce the first curve. Then, the aortic rings were washed with Kreb's buffer three times, allowed to stabilise, then added again with 100 μM ACh for 50 min in pre-contracted rat-aorta before adding extracts cumulatively to obtain the second curve.

were washed with Kreb's buffer three times, allowing the basal tension signal to stabilise for 10 min. The rings were pre-contracted again with 50 nM of NA followed by addition of 100 μM of ACh before adding 10 μg/mL kaempferol at 10 min interval to generate the second response curve.

Statistical analysis

Calculations and statistical analysis were performed using GraphPad Prism (Version 6.0) software. Contractile responses were expressed in grams of tension (g). The vasorelaxation due to ACh, the addition of extracts and kaempferol 3-O-rutinoside,

was expressed as a percentage relaxation in response to contraction caused by NA. All results were expressed as mean $\pm$ standard error of the mean (SEM) per experimental group with n representing the number of aortae used in each experiment. Data were analysed using two-way analysis of variance (ANOVA), which was followed by Dunnett's test for significant differences between the control and the experimental treatments, except for the data of kaempferol 3-O-rutinoside with the addition of Ach, which was analysed using unpaired Student's t-test. The line graph for determining the potent vasodilator between the two extracts was constructed using linear regression. P-values of less than 0.05 ($P < 0.05$) were considered statistically significant.

Results

MEGP produced higher percentage yield with more flavonoids and flavonols than AEGP

The percentage yield extracted from powdered leaves of GP using aqueous extraction (AEGP) or methanol extraction (MEGP) is shown in Table 1. After drying the extracted fluid, MEGP gave a higher percentage yield of 8.7%, producing a dark greenish-brown sticky solid material, while AEGP yielded 5.5%, generating a light brown fluffy material. The phytochemical analysis is presented in Table 2, which shows that flavonoids

Table 1. Percentage yield of aqueous extract of *Gynura procumbens* (AEGP) and methanoic extract of *Gynura procumbens* (MEGP).

Extract type	Description of the solid extract obtained	Yield (%)
AEGP	A light brown fluffy material	5.5
MEGP	A dark greenish-brown sticky solid material	8.7

Table 2. Phytochemical screening of aqueous extract of *Gynura procumbens* (AEGP) and methanoic extract of *Gynura procumbens* (MEGP).

Constituents	Specific test	Results	
		Aqueous	Methanol
Alkaloids	Dragendorff's test	-	-
	Mayer's test	+	-
Flavonoids	Sodium hydroxide test	+	+
Glycosides	Borntrager's test	-	-
Saponins	Frothing test	-	+
Steroids	Salkowski test	-	+
Tannins	Potassium dichromate test	-	+++
Triterpenoids		+++	++

+: weak presence, ++: moderate presence, +++: strong presence, and -: Absent.

and triterpenoids were detected in both AEGP and MEGP while saponins, steroids and tannins were only seen in MEGP. On the other hand, alkaloids were only detected in AEGP but not MEGP. Glycosides were absent in all extracts. Table 3 presents the total phenolic, flavonoid and flavonol contents in both types of GP extracts stated as mg GAE/g extract for total phenolic content and mg QE/g extract for total flavonoid and flavonol contents. AEGP has higher total phenolic content of 28.23 ± 4.68 mg GAE/g extract, which is twice as that of MEGP (13.82 ± 2.42 mg GAE/g extract). In contrast, MEGP has four times more total flavonoid (10.37 ± 0.07 mg QE/g extract) and two times more total flavonol (4.72 ± 0.04 mg QE/g extract) content than AEGP (2.52 ± 0.21 and 2.35 ± 0.03 mg QE/g extract, respectively). The total phenolic content of GP extracts was based on the standard gallic acid curve obtained at $y = 0.4332x + 0.0339$ ($r^2 = 0.993$), while the total flavonoid and flavonol contents were calculated from a standard quercetin curve using the linear regression equations $y = 0.0029x + 0.0458$ ($r^2 = 0.995$) and $y = 0.0045x + 0.039$ ($r^2 = 0.996$), respectively.

Kaempferol 3-O-rutinoside was detected in both extracts at a higher percentage in MEGP than AEGP

The results of HPLC screening using a reverse-phase C-18 column with a gradient system of acetic acid-acetonitrile combination as the mobile phase for both extracts revealed that when using the spiking technique with the standard reference compound of 100 $\mu\text{g/mL}$ kaempferol 3-O-rutinoside, the height of peaks for both extracts was explicitly increased with the retention time observed at 1.4 min after injection. The amount of kaempferol 3-O-rutinoside calculated based on the difference of the height of peaks at 1.4 min between the standard reference and the extracts were 57.86 $\mu\text{g/mL}$ and 78.35 $\mu\text{g/mL}$ for AEGP and MEGP, respectively. Figure 2 shows the actual HPLC chromatograms for AEGP and MEGP, with and without spiking of 100 $\mu\text{g/mL}$ kaempferol 3-O-rutinoside as the standard reference.

Both extracts mediated relaxation in aortic rings pre-contracted with NA in organ bath studies

Both AEGP and MEGP extracts were cumulatively added at 10 min interval in increasing concentrations (0.25, 0.5, 1 and 2 mg/mL). Addition of these extracts started at 20 min after the aortic rings were pre-contracted with 50 nM of NA. At the lowest concentration, both AEGP and MEGP produced a

Table 3. Total phenolic, flavonoids and flavonol contents of aqueous extract of *Gynura procumbens* (AEGP) and methanoic extract of *Gynura procumbens* (MEGP). Values are expressed as mean of mg of GAE or QE per gram of extract $\pm$ S.E.M; n=4.

	Total phenolic content	Total flavonoid content	Total flavonol content
AEGP	28.23 ± 4.68	2.52 ± 0.21	2.35 ± 0.03
MEGP	13.82 ± 2.42	10.37 ± 0.07	4.72 ± 0.04

GAE: Gallic acid equivalent, QE: Quercetin equivalent, *G. procumbens*: *Gynura procumbens*, SEM: Standard error of the Mean.

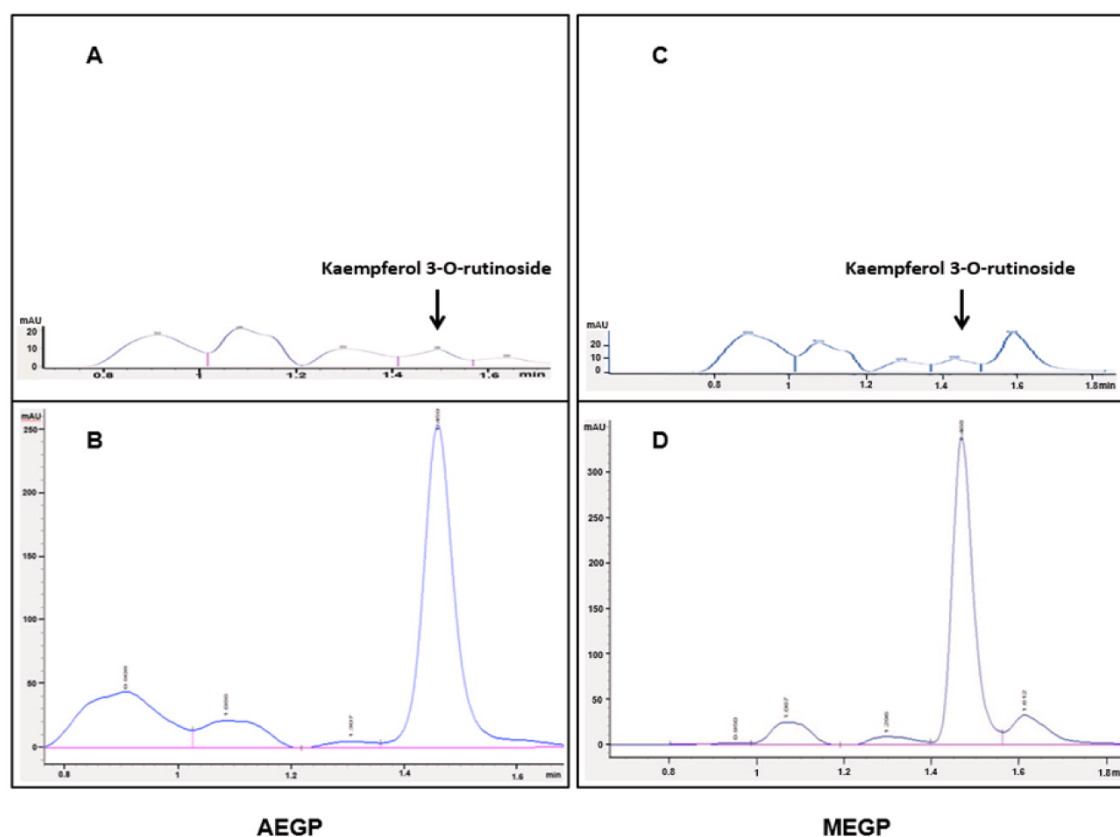


Figure 2. High-performance liquid chromatogram for aqueous extract of *Gynura procumbens* (AEGP) and methanoic extract of *Gynura procumbens* (MEGP). (A) AEGP alone with no spiking. (B) AEGP with spiking using 100 µg/mL of kaempferol-3-O-rutinoside standard reference stock. (C) MEGP alone with no spiking. (D) MEGP with spiking using 100 µg/mL of kaempferol-3-O-rutinoside stock. Detection of kaempferol 3-O-rutinoside was performed at 367 nm; heights of peaks were increased specifically at 1.4 min retention time when spiked with kaempferol 3-O-rutinoside (B and D) as compared with those without spiking (A and C).

minimum relaxation of $12 \pm 4.46\%$ and $18 \pm 3.22\%$, respectively (Figure 3). At the highest concentration, both AEGP and MEGP produced a maximum relaxation of $95 \pm 4.53\%$ and $112 \pm 6.07\%$, respectively (Figure 3). In the pattern of relaxation induced by both extracts, the percentage relaxation of NA-induced contraction in rat aorta was significantly higher when exposed to increasing concentration of GP extracts, especially from 0.5 mg/mL to 2 mg/mL for both extracts, as compared with that of the control NA alone (Figure 3).

Both extracts enhanced ACh-mediated response in intact and denuded endothelium rat aorta

In endothelium-intact rat aorta, both AEGP and MEGP extracts were cumulatively added at 10 min interval in increasing concentrations (0.25, 0.5, 1 and 2 mg/mL) at 10 min interval, starting at 20 min after adding 100 µM of ACh. Addition of these extracts started at 30 min following addition of 100 µM of ACh. At the lowest concentration, both AEGP and MEGP produced the minimum relaxation of $61 \pm 13.80\%$ and $72 \pm 18.40\%$, respectively (Figure 4A). At the highest concentration, both AEGP and MEGP produced the maximum relaxation of $87 \pm 4.30\%$ and $112 \pm 12.3\%$, respectively (Figure 4A). Relaxation

induced by both extracts was significantly different from ACh control alone, especially at 0.5 mg/mL for AEGP and 1 mg/mL for both AEGP and MEGP (Figure 4A).

In endothelium-denuded rat aorta, both AEGP and MEGP extracts were separately added in increasing and cumulative concentrations (0.25, 0.5, 1 and 2 mg/mL) at 10-min intervals. In both cases, the addition of these extracts started at 30 min following addition of 100 µM of ACh. At the lowest concentration, both AEGP and MEGP produced the minimum relaxation of $46 \pm 15.70\%$ and $42 \pm 13.10\%$, respectively (Figure 4B). At the highest concentration, both AEGP and MEGP produced the maximum relaxation of $79 \pm 5.60\%$ and $114 \pm 8.60\%$, respectively (Figure 4B). Overall, there was a pattern of relaxation induced by both extracts, where AEGP significantly enhanced relaxation at 1 mg/mL, while MEGP enhanced relaxation at a wider range of 0.5 mg/mL to 2 mg/mL (Figure 4B).

MEGP was more potent vasodilator than AEGP

Exposure of endothelium-intact rat aorta to either extract when added cumulatively produced a similar vasodilatory effect as a reduction in the percentage of NA peak response.

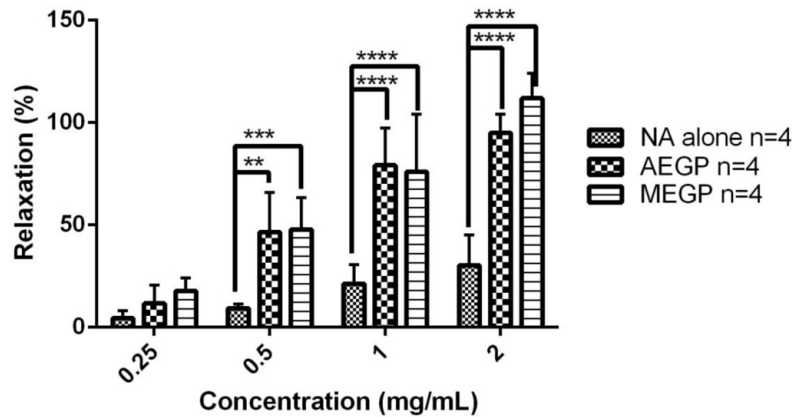


Figure 3. The percentage relaxation induced by noradrenaline (NA) in rat aortic rings in the absence or presence of aqueous extract of *Gynura procumbens* (AEGP) or methanoic extract of *Gynura procumbens* (MEGP) added cumulatively in increasing concentration (0.25, 0.5, 1 and 2 mg/mL) at 10 min interval (dt= 30, 40, 50 and 60 min). Data presented as mean $\pm$ S.E.M. The n values for NA and both extracts are 4. ** $P < 0.05$ for 0.5 mg/mL AEGP, *** $P < 0.05$ for 0.5 mg/mL MEGP and **** $P < 0.05$ for both 1 mg/mL and 2 mg/mL of AEGP and MEGP as compared with that of the control, NA alone.

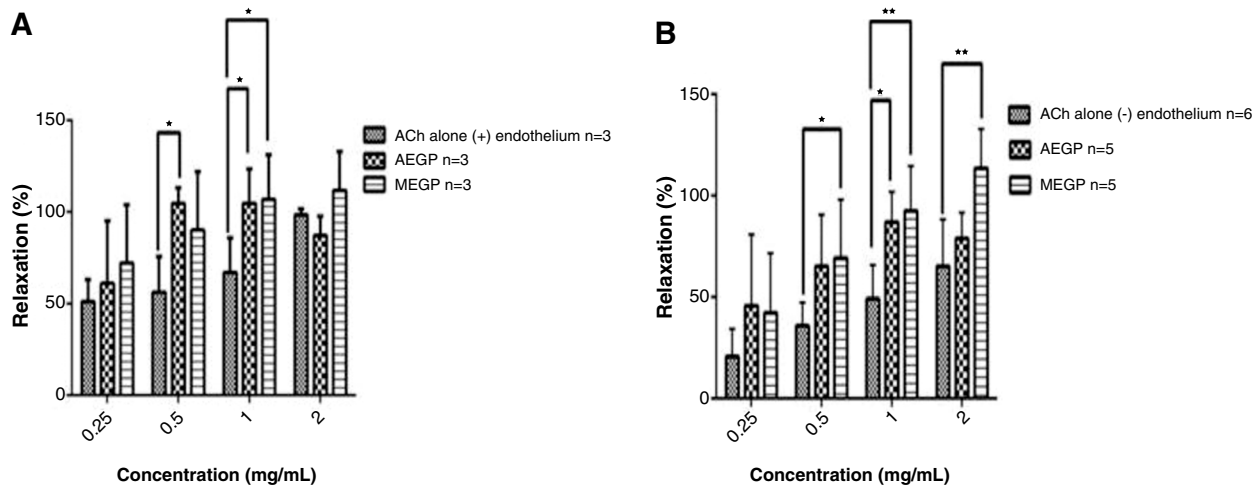


Figure 4. The percentage relaxation induced by acetylcholine (ACh) in the absence or presence of *Gynura procumbens* (AEGP) or methanoic extract of *Gynura procumbens* (MEGP) added cumulatively in increasing concentration (0.25, 0.5, 1 and 2 mg/mL) at 10 min interval (dt= 30, 40, 50 and 60 min). (A) Endothelium-intact rat aorta (B) Endothelium-denuded rat aorta. Data presented as mean $\pm$ S.E.M. The n values for ACh and both extracts are 3 for endothelium-intact rat aorta whereas n values for ACh and both extracts are 6 and 5, respectively, in endothelium-denuded aorta. * $P < 0.05$ for 0.5 mg/mL AEGP and 1 mg/mL both AEGP and MEGP as compared with the non-treated control, ACh alone in endothelium-intact rat aorta whereas * $P < 0.05$ for 0.5 mg/mL MEGP and 1 mg/mL AEGP as compared with non-treated control, ACh alone, and ** $P < 0.05$ for 1 mg/mL and 2 mg/mL MEGP vs. control in endothelium-denuded rat aorta.

Exposure to increasing concentration of AEGP up to 1 mg/mL reduced the contraction of rat aorta from $39 \pm 13.9\%$ to $0 \pm 17.1\%$, and MEGP from $40 \pm 18.4\%$ to $5 \pm 14.0\%$. ACh alone without the addition of extracts still showed a $23 \pm 4.90\%$ contraction. However, at 2 mg/mL, both extracts had a differential effect, in which MEGP caused further reduction from $5 \pm 14.0\%$ to $0 \pm 12.3\%$ contraction, whereas AEGP produced a rise of contraction from $0 \pm 17.1\%$ to $18 \pm 10.2\%$.

Exposure of endothelium-denuded rat aorta to either extract also produced a similar vasodilatory effect, in which the percentage

contraction of NA peak response at concentrations reaching up to 1 mg/mL was reduced from $65 \pm 9.35\%$ to $0 \pm 6.60\%$ for AEGP and $72 \pm 13.1\%$ to $21 \pm 9.88\%$ for MEGP, respectively. In comparison, ACh alone without the addition of any extract was only able to reduce the contraction from $80 \pm 9.31\%$ to $50 \pm 7.58\%$. However, at 2 mg/mL both extracts had a different effect in which MEGP caused a further reduction of contraction from $21 \pm 9.88\%$ to $0 \pm 8.64\%$, whereas AEGP produced a rise of contraction from $0 \pm 6.60\%$ to $8 \pm 5.61\%$. Overall, Figure 5 demonstrates the vasodilatory action of both GP extracts, expressed as the normalised data for the

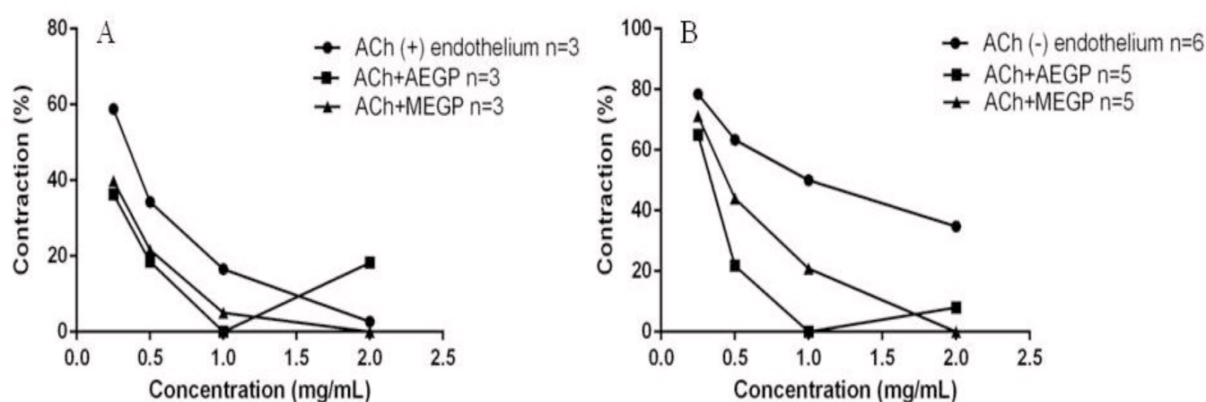


Figure 5. Normalised data for the effects of both *Gynura procumbens* (AEGP) and methanoic extract of *Gynura procumbens* (MEGP) expressed as the percentage of NA peak response. (A) Effect of both AEGP and MEGP in endothelium-intact aortic rings. (B) Effect of both AEGP and MEGP in endothelium-denuded aortic rings. Each extract (AEGP or MEGP) was cumulatively added in increasing concentration of 0.25, 0.5, 1, and 2 mg/mL. The effect of control (ACh alone) was measured at 30, 40, 50 and 60 min, corresponding to the time at which the respective concentration of extracts was added cumulatively. Data presented as mean $\pm$ SEM (n=3 for control and both AEGP and MEGP in endothelium-intact rat aortic rings; and, n=6 for control, and n=5 for both AEGP and MEGP, respectively, in endothelium-denuded rat aortic rings).

effect of both AEGP and MEGP observed as a reduction in the contraction of endothelium-intact and denuded aortic rings that were pre-contracted with 50 nM of NA.

Kaempferol 3-O-rutinoside reduced NA-mediated contraction

To elucidate the role of kaempferol-3-O-rutinoside in inducing relaxation, we first determined the vasodilatory effect of kaempferol without the application of ACh in the pre-contracted aorta rings. Kaempferol 3-O-rutinoside was added for 20 min, which started at 10 min after pre-contraction with 50 nM of NA. Addition of 10 μ g/mL of kaempferol 3-O-rutinoside caused rapid relaxation (Figure 7), which was significantly increased from $9.8 \pm 1.56\%$ to $84.9 \pm 18.7\%$ at 20 min and from $17.5 \pm 5.24\%$ to $94.6 \pm 5.59\%$ at 30 min, respectively, as illustrated in Figure 6.

Kaempferol 3-O-rutinoside enhanced the ACh-mediated response in endothelium-intact and -denuded rat aorta

The pre-contracted aortic rings with intact and denuded endothelium were supplemented with 10 μ g/mL of kaempferol 3-O-rutinoside at 20 min after addition of 100 μ M of ACh to determine the involvement of kaempferol 3-O-rutinoside in potentiating the cholinergic pathway. Addition of kaempferol 3-O-rutinoside significantly enhanced the vascular relaxation evoked by ACh in rat aorta with and without the endothelium. Kaempferol 3-O-rutinoside elicited a maximum relaxation of $76.0 \pm 4.66\%$ and $70.7 \pm 0.73\%$ with or without endothelium, respectively. In comparison, the control produced relaxation of $41.7 \pm 6.47\%$ and $19.9 \pm 6.69\%$, with and without endothelium, respectively (Figure 7).

Raw data for each experiment performed in this study are available, see *Underlying data*³⁰.

Discussion

Various studies have been carried out to identify the antihypertensive ability of GP extracts^{8–10,13–16}. The presence of bioactive compounds in GP is believed to contribute to its beneficial properties such as flavonoids, phenolic acids, alkaloids, terpenoids, glycosides and sterols¹². In this present study, the antihypertensive property of both crude extracts of AEGP and MEGP was investigated *in vitro* to determine whether they were able to potentiate the cholinergic mediated vasodilation. Limited studies showed the possible contribution of the cholinergic pathway in enhancing the relaxation effect of GP extracts. Successful extractions with water or methanol from the dried and powdered leaves of GP were achieved, producing the fluffy brown powder and the sticky dark greenish-brown substance as yields for AEGP and MEGP, respectively. The characteristics of AEGP in our study were similar to those reported by Lee *et al.*²³ and Kamaruzaman and Mat Noor³¹. There was neither an established guideline nor any known protocols for methanol-based extraction for GP plant leaves. However, the characteristics of the dry methanol extracts, yielding a dark brown sticky solid was demonstrated by Korwar *et al.*³², similar to our findings. The percentage yields for both extracts were different, which might be due to the polarity of the solvents used³³. Given an equal initial amount of fresh weight for the plant parts, the aqueous solvent yielded less extract than those extracted with the methanol solvent, suggesting that the plant GP possesses more methanol-soluble components³³. Our finding is in concordance with a study done by Njume *et al.*³⁴ and Ngo *et al.*³⁵, where absolute methanol or a mixture of 50% (v/v) water with other solvents such as alcohol or acetone was recommended as the solvent of choice to produce high levels of extractable ingredients.

Our results highlighted the observation that both extracts, which were obtained using different solvents, contained

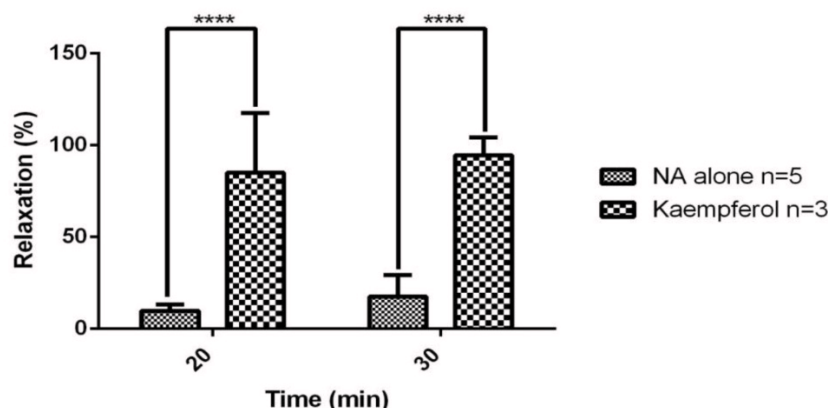


Figure 6. The percentage of relaxation induced by kaempferol 3-O-rutinoside in the pre-contracted rat aorta in comparison with noradrenaline (NA) alone. Data presented as mean $\pm$ SEM. The n values for NA alone and NA with kaempferol 3-O-rutinoside are 5 and 3, respectively. **** $P < 0.05$ for the addition of kaempferol 3-O-rutinoside at 20 and 30 min vs control, NA alone.

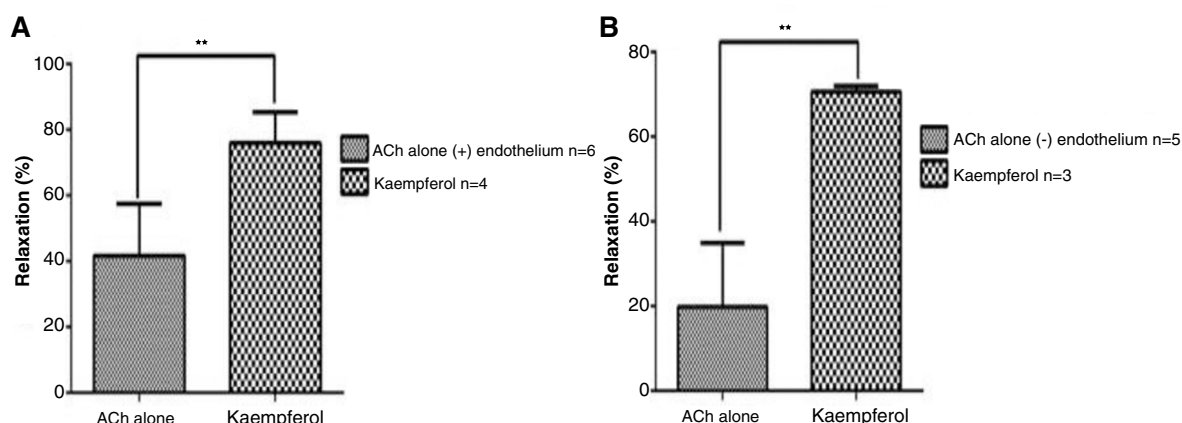


Figure 7. The percentage relaxation induced by acetylcholine (ACh) in the absence or presence of 10 $\mu\text{g/mL}$ of kaempferol 3-O-rutinoside in endothelium-intact rat aortic rings pre-contracted with noradrenaline. (A) Endothelium-intact rat aorta (B) Endothelium-denuded rat aorta. Data presented as mean $\pm$ SEM. The n values for ACh alone and kaempferol 3-O-rutinoside are 6 and 4, respectively in endothelium-intact rat aorta. In contrast, the n values for ACh alone and kaempferol 3-O-rutinoside are 5 and 3, respectively in endothelium-denuded rat aorta. ** $P < 0.05$ for the addition of kaempferol 3-O-rutinoside at 30 min vs control, ACh alone in both endothelium-intact and -denuded rat aorta.

phytochemicals, such as flavonoids and triterpenoids. In addition, MEGP responded positively for the saponins, steroids and tannins, while AEGP was detected positive for alkaloids. However, both extracts did not give a positive test for glycosides. The efficiency of methanol in extracting phytochemicals has been reported in the previous studies^{34,36,37}. Our results were consistent with those of others, confirming that methanol is an appropriate solvent for extracting bioactive compounds from GP, giving higher yields than its aqueous counterpart.

Phenolic compounds account for the majority of pharmacological actions. For several decades, phenolic compounds are regarded as having a powerful free radical scavenging activity^{38,39}. As many aspects of CVDs are associated with high oxidative stress, the consumption of polyphenol-rich food that combats oxidative stress can help lower the risk of chronic diseases,

including cardiovascular disorders^{40–42}. In this present study, the total phenolic content of AEGP is higher than MEGP. However, the latter was shown to contain more total flavonoids and total flavonol content. The results obtained for the flavonoids are in agreement with the previous report on GP by Afandi *et al.*²², showing that MEGP contains higher total flavonoid content than AEGP, but the amount of phenolic content was found lowest in AEGP among all the other extract preparations. This variation could be due to differences in the process of extractions and the solvents used. Various factors could contribute to the efficiency of extraction, such as the type and concentration of solvents, pH, time, temperature and solid-liquid ratios⁴³. Besides, the different types and amount of phenolic and non-phenolic compounds extracted are dependent on the extraction solvent used⁴⁴. The results showing that AEGP contained a lower percentage of total flavonoid and total flavonol compounds

than MEGP might be due to flavonoids having low solubility in water²⁰.

The presence of flavonoids in both extracts suggests they have a considerable role in various pharmacological effects, especially the hypotensive action, which might explain the BP- lowering observation and claims in the traditional practice of consuming the GP leaves to treat hypertension¹⁹. Experimental evidence has shown that the flavonoids in GP extracts is responsible for an antihypertensive effect^{10,14}. Kaempferol, which is one of the significant flavonoids dominantly present in the sub-fraction of the ethanolic extract of GP^{14,20,21}, was also detected in both extracts in our study. Kaempferol 3-O-rutinoside was detected in a higher amount from MEGP than AEGP. After quantifying this active ingredient, we decided to test its vasodilatory activity in the organ bath system using rat aorta to elucidate its hypotensive contribution. We first added the extracts after NA-induced contraction of aortic rings to confirm the vasodilatory action of both extracts. Our results highlighted the vascular relaxation effect indicating the antihypertensive property of this plant and confirming reports from previous studies that GP extracts might reduce blood pressure by vasodilation^{8–10,13,14,45}. Next, we investigated whether there is a possible contribution of the cholinergic-mediated pathway in the vasodilatory effect of the extracts. Our present finding demonstrated that the vasorelaxation activity of the extracts further enhanced the ACh-mediated vasodilation in the endothelium-intact aorta, primarily upon exposure to 0.5 mg/mL AEGP and 1 mg/mL of both AEGP and MEGP. These results support the findings of Kaur *et al.*⁸, which showed that ACh administration to GP-treated rats *in vivo* significantly reduced the mean arterial pressure (MAP) and diastolic pressure (DP) of spontaneously hypertensive rat (SHR), and that of Kim *et al.*¹³, which detected an increased of NO levels in the serum of *Gynura procumbens* water extract (GPWE)-treated SHR.

Our data further revealed that the extracts significantly exerted their relaxation effect in the endothelium-denuded aorta, suggesting that the vasodilatory action of AEGP and MEGP extracts is also mediated directly through the vascular smooth muscles. The vascular smooth muscle cells are located within the media of the vascular bed, directly modulating the vascular tone primarily via the changes in sarcoplasmic free Ca^{2+} concentration⁴⁶. Various signalling mechanisms could trigger the response of the smooth muscles through an ACh endothelium-independent pathway such as the adrenergic pathway. In the adrenergic pathway, α_1 and α_2 adrenoceptors mediate contraction of the smooth muscles by coupling to Gq and activating phospholipase C (PLC), thereby increasing the concentration of Inositol 1,4,5-triphosphate (IP_3), which in turn, causes the rise in intracellular Ca^{2+} concentration⁴⁷. A previous study showed that GP extracts could mediate relaxation via α_1 receptors *in-vivo*⁸. PE (an α adrenoceptor agonist) and methoxamine (a selective α_1 adrenoceptor agonist) induced a significant decrease in presor response of GPWE-treated rats⁸. From our findings, we speculate that the GP extracts might also mediate relaxation via adrenergic pathway by activating α and β adrenoceptors. A more favored explanation for this action, however, is based on the evidence that we obtained so far, which pointed towards the cholinergic pathway. It is known that ACh fails to induce relaxation when the endothelium is denuded and removed^{48,49},

suggesting that vasorelaxation of smooth muscle is inhibited when the formation of NO is impaired due to activation of M_2 and M_3 receptors, hence causing contraction instead^{50,51}. Thus, we focused more on investigating the cholinergic endothelium-independent pathway via stimulation of M_2 and M_3 muscarinic receptors that inhibit the contraction. Overall, our follow-up study elucidated and confirmed that the underlying mechanism of action for both extracts was mediated via the cholinergic pathway, with a significant contribution from kaempferol.

Investigation on the vasodilators between these two extracts was carried out in both endothelium-intact and endothelium-denuded rat aorta. Results have shown that upon exposure at the same concentration of 2 mg/mL, MEGP produced a more potent vasodilatory effect than that of AEGP. Although at a lower concentration, both extracts responded in a dose-dependent manner, AEGP seemed to reverse the effect into slight contraction upon reaching the concentration of 2 mg/mL, unlike MEGP, which continued to cause further relaxation. Drug effects are caused by the interaction of the drug molecules and the receptor. At low concentration of the drug, many binding sites are still available; however, further increasing the drug concentration causes more drug-receptor complex formation and a substantial increase in drug effect⁵² until all of the receptor binding sites are occupied. Further increasing the drug concentration causes receptors to become saturated, leading to no or little effect of the drug⁵². Thus, the slight reverse effect of contraction by AEGP at 2 mg/mL indicates the possible saturation of all the receptors binding sites for the relaxation effect of the blood vessels, limiting any further vasodilation. Thus, in using AEGP, a possibly saturated concentration exceeding 2 mg/mL would not cause any relaxation effect but a contraction of the blood vessels instead.

Flavonoids rich food, primarily via consumption of botanicals, is often associated with a reduction of cardiovascular diseases^{53–55}. Kaempferol 3-O-rutinoside was detected and quantified as one of the active ingredients in GP²⁰. Thus, we decided to test its hypotensive property in an organ bath system using rat aorta. We first carried out the experiments to confirm the relaxant effect of kaempferol 3-O-rutinoside in rat aortic rings, resulting in vasodilatory activity after NA-induced contraction. This result is in agreement with those of previous studies that demonstrated the vasodilatory effects of kaempferol in different vascular beds^{55–58}. Similar findings have also been reported on the vasorelaxant properties of other flavonoids such as quercetin, myricetin, rutin and apigenin^{57,59}. Kaempferol, however, is considered as the most effective among all flavonoids in potentiating vasorelaxation, thus might have more significant potential in preventing and treating CVDs⁵⁷. We assessed the involvement of kaempferol 3-O-rutinoside in potentiating ACh-dependent endothelium-mediated vasodilation, and ACh-independent mediated contraction. Our results showed that kaempferol elicited the relaxation response produced by ACh in both endothelium-intact and endothelium-denuded rat aorta. Our observation suggests that kaempferol enhanced the relaxation in rat aortic rings possibly via endothelium-dependent and endothelium-independent cholinergic pathway, following previous reports^{57,58}, which is possibly mediated by kaempferol 3-O-rutinoside, as illustrated in [Figure 8](#).

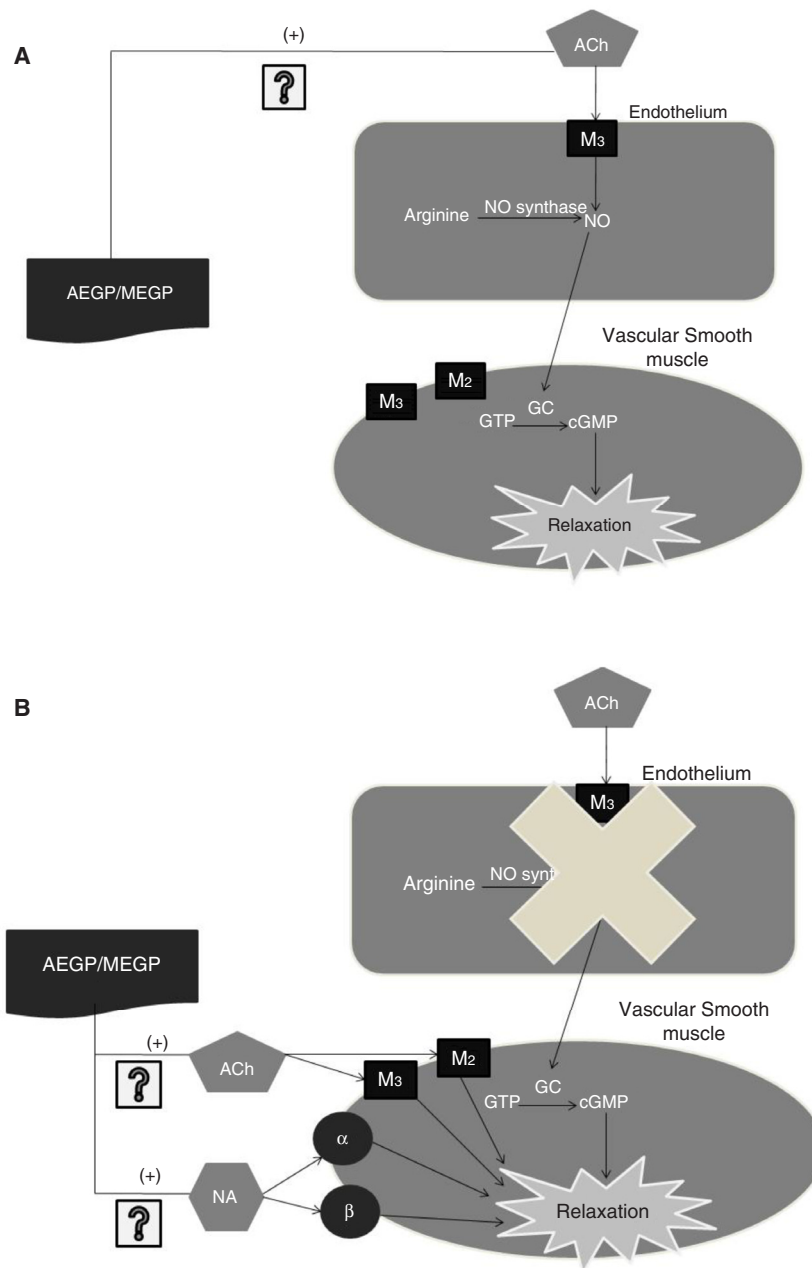


Figure 8. Effect of extracts in endothelium and smooth muscle cells of rat aorta possibly via activation of the endothelium-dependent cholinergic pathway or directly via smooth muscle. The figure is a simplified representation showing the main component associated with acetylcholine mediated endothelium vasodilation in the isolated rat aorta (IRA) model and showing the involvement of both extracts in inducing relaxation in vascular endothelium or vascular smooth muscle. In the presence of endothelium, both extracts (AEGP/MEGP) mediate vasodilation possibly by acting on the muscarinic ACh receptor M₃ by producing NO. When the endothelium is disrupted, the extracts mediate relaxation on vascular smooth muscle possibly via endothelium-independent cholinergic pathway by acting on the muscarinic ACh receptor M₂ and the muscarinic ACh receptor M₃ or via adrenergic pathway by acting on α and β adrenoceptors. The vasorelaxant effect produced by the extracts is mediated by kaempferol, particularly, kaempferol 3-O-rutinoside. ACh; Acetylcholine M₂; Muscarinic acetylcholine receptor M₂; M₃; Muscarinic acetylcholine receptor M₃; α: alpha adrenoceptors; β: beta-adrenoceptors; NO: Nitric oxide; GC: Guanylyl cyclase; cGMP: cyclic guanosine monophosphate; AEGP: Aqueous extract of *G. procumbens*; MEGP: Methanol extract of *G. procumbens*.

Summary and conclusion

The result of this study shows that crude extracts of AEGP and MEGP possessed different characteristics and physical appearance,

as well as the percentage yield. MEGP was a dark greenish-brown sticky solid whereas AEGP was a light brown fluffy material. The percentage yield of MEGP was higher than AEGP.

The result of this study also reveals the presence of essential phytoconstituents in both AEGP and MEGP, and MEGP was found to contain more active constituents than AEGP. Besides, MEGP also contained higher total flavonoid and flavonol contents than AEGP, although the amount of phenolic content was lower in MEGP. HPLC study has demonstrated the presence of kaempferol 3-O-rutinoside, where it is higher in MEGP than AEGP. Treatment with the pure active ingredient kaempferol 3-O-rutinoside relaxed both tissue preparations. Overall, these results imply the possible involvement of extracts in the cholinergic pathway, which is possibly mediated by kaempferol 3-O-rutinoside, as shown by its vasorelaxation effects in endothelium-intact and endothelium-denuded aorta. The present finding also demonstrates the vasodilatory effect of the two extracts, in thoracic aorta tissues isolated from rats, possibly via the contribution of the cholinergic mediated pathway. It is also concluded

that the main active vasodilatory component in both extracts causing such hypotensive effect is the flavonoid particularly kaempferol 3-O-rutinoside. However, further study is necessary to elucidate its mechanism of action.

Data availability

Underlying data

Open Science Framework: Anti-hypertensive Vasodilatory Action of *Gynura procumbens*. <https://doi.org/10.17605/OSF.IO/W6XJV>³⁰

This project contains “Anti-hypertensive Vasodilatory Action of *Gynura procumbens*.xlsx”, which includes raw data for all experiments performed in this study.

Data are available under the terms of the Creative Commons Attribution 4.0 International licence (CC-BY 4.0).

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Yun Shin Sew 

Malaysian Agricultural Research & Development Institute (MARDI), Seri Kembangan, Malaysia

This article by Shahlehi *et al.* reported interesting findings about the vasodilatory effects of two different plant extracts of *Gynura procumbens*. The authors have shown the one extracted using methanol solvent, MEGP contains higher amount of flavonoids particularly kaempferol 3-O-rutinoside than the water solvent, AEGP. Following that, the authors showed MEGP works better for *in vitro* vasodilatory performance than AEGP. The authors also claim a probable role of kaempferol 3-O-rutinoside in both extracts as the main causal factor via experimental observations. There are several comments on this article as the followings:

1. The authors should state the volume of individual AEGP and MEGP used for HPLC analysis in the material and method section.
2. Table 1 should be removed as those are simple information which could be just included in the text.
3. There are discrepancies in the usage of asterisk mark for statistical significance level (referring to Figure 3, 4 and 7) which might confuse the readers. Single asterisk often indicate $P < 0.05$ and double asterisk for $P < 0.01$, need to standardize the usage of asterisk(s) in all figures.
4. It was a good experimental design in such a way that the relaxant effect of kaempferol 3-O-rutinoside in rat aortic rings was investigated. However the amount of kaempferol 3-O-rutinoside tested was pretty low (10ug/mL). Could this be tested with higher amount of kaempferol 3-O-rutinoside since as high as 57.86 ug/mL and 78.35 ug/mL of this compound was found in AEGP and MEGP extract, respectively? Given that higher concentration of extract showed adverse effect of vasodilatory action in Figure 5, will any adverse effect happen when higher kaempferol 3-O-rutinoside is being used (mimicking the concentration of the compound found in each extract)?
5. Just an additional suggestion for the future works of this study. Though the methanol extract of *Gynura procumbens* performs better than the one extracted using water (as shown

in the present study), methanol extracts of herbs are often considered to be more toxic than water extracts. Therefore, it is necessary to investigate the safety level of this methanol extract. A follow-up *in vivo* study using rat model should be conducted in future.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular and safety analysis of food and herb products

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 13 October 2020

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Adi Idris 

Menzies Health Institute Queensland, School of Medical Science, Griffith University, Gold Coast, QLD, Australia

Shahlehi et al. reports the vasodilatory effects of the active flavonoid component of *Gynura procumbens*, kaempferol 3-O-rutinoside, on rat aortic rings. Though the findings are interesting, it is difficult to see the novelty in this work compared to those done by others. Authors are advised to highlight the novelty of their work. Some other comments that authors are recommended to

address are listed below.

Background:

- Authors are asked to comment on reference made to Abrika et al 2013, where they looked at the effect of GP flavonoid fraction on rat arterial contraction as I am contending lack of novelty based on this.

Results:

- Tables 1 and 3 are unnecessary. Information could easily be stated in text without the need of a table.
- Figure 5 is the critical figure in this manuscript. However, error bars are not visible. Is there a reason why there is only $n=3$ for each treatment arm in figure A and $n>5$ in figure B? Authors are also asked to keep Y-axis scale consistent between both figures.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Cell biology and drug delivery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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