Research Paper

Vasorelaxant effect of water fraction of *Labisia Pumila* and its mechanisms in spontaneously hypertensive rats aortic ring preparation

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ABSTRACT

Introduction: *Labisia pumila* has been reported to possess activities including antioxidant, anti-aging and anti-cancer but there is no report on its vasorelaxant effects.

Objective: This study aims to fractionate water extract of *Labisia pumila*, identify the compound(s) involved and elucidate the possible mechanism(s) of its vasorelaxant effects.

Methods: Water extract of *Labisia pumila* was subjected to liquid-liquid extraction to obtain ethyl acetate, n-butanol and water fractions. In SHR aortic ring preparations, water fraction (WF-LPWE) was established as the most potent fraction for vasorelaxation. The pharmacological mechanisms of the vasorelaxant effect of WF-LPWE were investigated with and without the presence of various inhibitors. The cumulative dose-response curves of potassium chloride (KCl)-induced contractions were conducted to study the possible mechanisms of WF-LPWE in reducing vasoconstriction.

Results: WF-LPWE produced dose-dependent vasorelaxant effect in endothelium-denuded aortic ring and showed non-competitive inhibition of dose-response curves of PE-induced contraction, and at its higher concentrations reduced KCl-induced contraction. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) significantly inhibited vasorelaxant effect of WF-LPWE. WF-LPWE significantly reduced the release of intracellular calcium ion (Ca2+) from the intracellular stores and suppressed the calcium chloride (CaCl2)-induced contraction. Nω-nitro-L-arginine methyl ester (L-NAME), methylene blue, indomethacin and atropine did not influence the vasorelaxant effects of WF-LPWE.

Conclusion: WF-LPWE exerts its vasorelaxant effect independently of endothelium and possibly by inhibiting the release of calcium from intracellular calcium stores, receptor-operated calcium channels and formation of inositol 1,4,5-triphosphate. WF-LPWE vasorelaxant effect may also mediated via nitric oxide-independent direct involvement of soluble guanylate cyclase (sGC)/ cyclic guanosine monophosphate (cGMP) pathways.

1. Introduction

*Labisia pumila* (*L. pumila*), a herbal plant from the family of Myrsinaceae, is locally called ‘Kacip Fatimah’, ‘Selusuh Fatimah’ and ‘Akar Fatimah’ [1]. Pharmacological studies have shown that *L. pumila* contains flavonoids and phenolic compounds [2], carotenoids, ascorbic acids, alkenyl and benzoquinone which have been reported to possess antioxidant, anti-inflammatory and anti-cancer properties [3]. The antioxidant property is useful to treat disease involving oxidative stress, such as cardiovascular disease [4]. *L. pumila* exhibited phytoestrogen activity and has benefit in modulating post-menopausal cardiovascular risk [5]. Scientific reports on the antihypertensive properties of *L. pumila* remain elusive and there is no report so far on its vascular activities. The present study aims to investigate the mechanisms of vasorelaxant effects of *L. pumila* extracts in spontaneously hypertensive rats.

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1 These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.
2. Materials and methods

2.1. Plant material

*L. pumila* was obtained from Pos Kuala Sungai Mu, Sungai Siput Utara, Perak, Malaysia in January 2016. Identification and authentication of the plant were carried out by Herbarium, School of Biological Sciences, Universiti Sains Malaysia (USM) (Specimen voucher registration no: 11632).

2.2. Crude extraction and fractionation of *L. pumila*

Dried aerial and leaf parts of *L. pumila* were ground and extracted by serial maceration using petroleum ether, chloroform, ethyl acetate, and methanol at 40 °C and distilled water at 50 °C for 24 h to obtain petroleum ether extract (LPEA), chloroform extract (LPCHELOR), ethyl acetate extract (LPEA), methanol extract (LPMEOH) and water extract (LPWE) of *L. pumila*. Macerations of each solvent were repeated thrice. Each extract was concentrated using rotary evaporator under vacuum and oven-dried at 40 °C and kept in a freezer until used. LPWE (20 g) was subjected to liquid-liquid fractionation following the earlier method by Samud et al. (1999) with some modifications [6]. Briefly, the extract was dissolved in 400 mL water and was then fractionated with 400 mL of ethyl acetate and n-butanol in separating funnel. The resultant ethyl acetate fraction (EAF-LPWE), n-butanol fraction (BF-LPWE) and the residual water fraction (WF-LPWE) were concentrated using rotary evaporator under vacuum and oven-dried at 40 °C and kept in a freezer until used. All fractions were freshly dissolved in normal saline prior to the experiment.

2.3. Experimental animals

Male spontaneously hypertensive rats (SHR, 250–300 g) were housed in individual cages with free access to food ad libitum and water and maintained at Animal Transit Facility of School of Pharmaceutical Sciences, USM. All procedures involving animals were conducted according to the guidelines by the USM Institutional Animal Care and Use Committee (USM IACUC). (USM/Animal Ethics Approval/2015/96)/(685).

2.4. Drugs, chemicals and solvents

Petroleum ether, chloroform, ethyl acetate, methanol, phenylephrine hydrochloride (PE), acetylcholine (ACh), indomethacin, and Nω-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma-Aldrich, Germany; while sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH2PO4), magnesium sulphate (MgSO4⋅7H2O), glucose, sodium hydrogen carbonate (NaHCO3), and calcium chloride dehydrate (CaCl2⋅2H2O) were purchased from R&M Chem., UK. All drugs were freshly prepared in normal saline, except indomethacin in 0.5% (w/v) sodium carbonate, prior to use.

2.5. Non-invasive blood pressure monitoring in conscious SHR by tail cuff method

After 1 week of acclimation, thirty six (36) SHRs were divided into six (6) groups and were fed orally for 28 days as follows: Group 1 received vehicle (5% Tween80 and served as negative control. Group 2 received verapamil (15 mg/kg) and served as the reference group. Group 3, 4, 5 and 6 received LPPE, LPCHLOR, LPMEOH and LPWE (500 mg/kg), respectively. Systolic blood pressure (SBP) were measured weekly by tail-cuff method using non-invasive blood pressure monitoring system, CODA (Kent Scientific Inc.) on day 0 (D0), day 7 (D7), day 14 (D14), day 21 (D21) and day 28 (D28).

2.6. Preparation of isolated rat thoracic aortic rings

The rat was anesthetized with urethane (1.3 g/kg, i.p.). A midline abdominal incision was performed to expose the aorta. The thoracic aorta was isolated, cleaned from the adherent fat and connective tissues, and cut into 3–5 mm rings. The aortic rings were then suspended horizontally in tissue chambers containing 10 mL of Kreb's solution (mmol/L: NaCl 118.6, KCl 4.1, CaCl2 2.5, MgSO4⋅7H2O 1.2, KH2PO4 1.2, NaHCO3 25.1, and glucose 11.0). The tissue-bath solution was bubbled incessantly with 95% O2 and 5% CO2 (carbogen) at 37 °C. Aortic rings were allowed to equilibrate at an optimal tension of 1 g for 45 min. Responses were recorded isometrically via a force transducer (Grass FT03D) connected to a computerized data acquisition system (PowerLab; ADInstruments Pty Ltd., Australia).

2.7. Effects of extracts of *L. pumila* and fractions of *L. pumila* water extract on isolated aortic rings precontracted with phenylephrine

Aortic rings were hanged in the organ chamber as described above. The tissues were precontracted with PE (1 µM). Upon attaining a stable plateau, 0.1 mL of LPPE, LPCHLOR, LPMEOH, and LPWE cumulatively added (0.2–4.0 mg/mL) into the organ bath, respectively. The tensions attained following the contraction induced by PE, and the concentration relaxation responses following cumulative additions of *L. pumila* extracts were recorded. The procedures were repeated for fractions of LPWE (WF-LPWE, EAF-LPWE and BF-LPWE, 0.1–4.0 mg/mL).

Based on the screening done with all three fractions, WF-LPWE had produced the largest relaxation of the aortic rings. Therefore, this fraction was selected for the mechanistic study.

2.8. Role of nitric oxide synthase (NOS), prostacyclin and muscarinic receptors on WF-LPWE induced relaxation

To determine the possible role of NOS, prostacyclin and muscarinic receptors in the vasorelaxant effects of the fraction, the intact aortic rings were preincubated with Nω-nitro-L-arginine methyl ester (L-NAME, 10 µM), indomethacin (10 µM) and atropine (10 µM) for 20 min, respectively, before precontracted with PE (1 µM). WF-LPWE (0.01 µg to 3.0 mg/mL) was then added cumulatively to the chamber at the plateau of the PE-induced contraction and the concentration-relaxation response curve was determined.

2.9. Role of cyclic guanosine monophosphate (cGMP) pathway on WF-LPWE induced relaxation

Endothelium-intact aortic rings were incubated with methylene blue (10 μM) and 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 µM) for 20 min before precontraction with PE (1 µM), respectively. Cumulative concentrations of WF-LPWE (0.01 µg - 3.0 mg/mL) were added into the chamber to determine the concentration-relaxation response curve.

2.10. Effect of WF-LPWE on KCl-induced contraction

To access the ability of WF-LPWE in reducing the vasoconstriction, the cumulative dose response curve of KCl-induced (10–90 mM) contraction were examined in the presence of WF-LPWE at 0.5, 1.0 and 2.0 mg/mL, respectively.

2.11. Effect of WF-LPWE on intracellular calcium release

To assess the effect of WF-LPWE on the mobilization of calcium ions (Ca2+) from intracellular stores, the denuded aorta were incubated in calcium-free Kreb's solution containing 1.5 mM/L of ethylene glycol tetraacetic acid (EGTA), PE (1 µM) was instilled to produce a steady-state contraction (con1). The second contraction (con2) was obtained after preincubating the baths with or without WF-LPWE for 10 min. The ratio of con2 over con1 was calculated.
2.12. Effect of WF-LPWE on calcium-induced vasoconstriction

To investigate whether the relaxation in the aortic rings involved calcium influx, the denuded aortic tissue was washed 4–5 times in calcium-free Kreb's solution and could stabilize. PE (1 μM) was added before incubation with WF-LPWE to produce a steady contraction. The tissue was then incubated with 0.5 mg/mL WF-LPWE for 15 min before the addition of cumulative dose of calcium (0.01–3.0 mM) to obtain the dose–response curve.

2.13. Reverse-phase high-performance liquid chromatography (HPLC) analysis of WF-LPWE

Stock solution of gallic acid and catechin (1 mg/mL in methanol) were diluted to the concentration range of 20.0–0.078 μg/mL. The analysis was conducted using Agilent HPLC 1260 system equipped with quaternary pump, UV detector, auto sampler, column heater, photodiode array detector, online degasser and Zorbax Eclipse Plus C18 reverse-phase column (4.6 × 250 mm, 5 μm). The mobile phase consists of A (0.25% phosphoric acid) and B (acetonitrile HPLC grade). The content of gallic acid and catechin were estimated by reverse-phase HPLC method described by Yusoff and Wan Mohamud (2011) with minor modifications [7]. The concentration of gallic acid and catechin in the samples were determined based on the regression lines of gallic acid and catechin in the range of 0.078–20.0 μg/mL, which were y = 68.43x + 8.881 (R² = 0.9997) and y = 5.4368x + 0.291 (R² = 0.9994) respectively. Y is the peak area of the analyte and X is the concentration of the analyte (μg/mL).

The method validation was performed according to the routine validation protocol. The precision was assessed by replicate analyses of samples at five different concentrations (0.039–20.0 μg/mL) and was expressed as percentage relative standard deviation (% RSD). Intra-day precision and accuracy was performed from the variability of the replicate analyses of samples (n = 5) in the same method run. The inter-day were expressed for three continuous days.

2.14. Data analysis

All data are given as mean ± standard error means (S.E.M.). Data were analysed using Student's t-test and also two-way ANOVA followed by Bonferroni’s post hoc test. All analyses were carried out using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., USA). Values of p < 0.05, p < 0.1, p < 0.01 and p < 0.001 were considered statistically significant.

3. Results

Our preliminary vasorelaxation evaluation using various extracts of L. pumila suggested that LPWE had produced the best pharmacological action. Therefore, LPWE was subjected to fractionation producing three fractions: WF-LPWE, EAF-LPWE and BF-LPWE. Further investigation was carried out to evaluate vasorelaxant effects of these fractions prior to possible mechanistic study.

3.1. Effect of L. pumila water extract on blood pressure

Verapamil (15 mg/kg) treated group showed significantly (p < 0.05) lower in SBP at D7 (D7 = 155.0 ± 14.2 mmHg vs. D0 = 202.2 ± 0.4 mmHg). LPWE (500 mg/kg) showed significantly (p < 0.05) lower in SBP at D28 (D28 = 164.5 ± 23.4 mmHg vs. D0 = 215.4 ± 6.7 mmHg).

3.2. L. pumila extracts and fractions of LPWE induce vasorelaxation in intact aortic ring of SHRs

All five extracts significantly enhanced (p < 0.001) the vasorelaxation of aortic rings as compared with control. LPWE produced significant vasorelaxation at the lowest dose (0.5 mg/mL), therefore, this extract was chosen to further fractionate. All three fractions significantly enhanced (p < 0.001) the vasorelaxation of the intact aortic rings as compared with control. WF-LPWE produced the largest vasorelaxant effect with significant relaxation at the lowest dose (0.25 mg/mL), therefore, this fraction was chosen for further mechanistic study (Fig. 1A and B) (see Table 1).

3.3. Vasorelaxant effect of WF-LPWE in intact and denuded aortic ring of SHRs

WF-LPWE (0.1-4.0 mg/mL) exhibited a dose-dependent vasorelaxant effect on PE-precontracted endothelium intact aortic rings with maximum relaxation (Rmax) value of 113.8 ± 3.1%. Vasorelaxant effect of WF-LPWE (0. 4.0 mg/mL) on endothelium-denuded aortic rings preparations showed significantly reduced in the relaxation response curve of WF-LPWE at the concentration of 0.5 mg/mL when compared to the control with the Rmax value of 174.3 ± 1.7% at 4.0 mg/mL (Fig. 1C and D) (see Table 1).

3.4. Involvement of NOS, prostacyclin and muscarinic receptor in WF-LPWE-induced relaxation

The vasorelaxant effects of WF-LPWE following incubation with L-NAME (10 μM), indomethacin (10 μM) and atropine (10 μM) did not show any significant decrease. WF-LPWE + L-NAME (Rmax = 81.6 ± 5.5%) vs. WF-LPWE (Rmax = 70.4 ± 7.6%), WF-LPWE + indomethacin (Rmax = 73.9 ± 13.4%) vs. WF-LPWE (Rmax = 80.0 ± 13.3%), WF-LPWE + atropine (Rmax = 79.1 ± 7.8%) vs. WF-LPWE (Rmax = 52.9 ± 8.8%), respectively (see Table 1).

3.5. Role of cyclic guanosine monophosphate (cGMP) pathways on in WF-LPWE-induced relaxation

Endothelium-intact aortic rings pre-incubated with methylene blue (10 μM) produced no significant change in the vasorelaxation. WF-LPWE + MB (Rmax = 98.4 ± 10.9%) vs. WF-LPWE (Rmax = 93.7 ± 5.6%) as observed in Fig. 2A. In Fig. 2B, endothelium-intact aortic rings pre-incubated with ODQ (10 μM) showed significant inhibition in vasorelaxation. WF-LPWE + ODQ (Rmax = 50.3 ± 4.4%) vs. WF-LPWE (Rmax = 87.5 ± 6.7%) (see Table 1).

3.6. Effect of WF-LPWE on adrenergic receptors

Incubation of aortic rings with both α1 inhibitor, prazosin and β2-inhibitor, propanolol (10 μM) pre-contracted with PE (1 μM) did not elicit any significant change in the vasorelaxation. WF-LPWE + prazosin (Rmax = 39.7 ± 10.0%) vs. WF-LPWE (Rmax = 53.6 ± 4.6%) and WF-LPWE + propanolol (Rmax = 65.7 ± 3.3%) vs. WF-LPWE (Rmax = 62.9 ± 2.3%) (see Table 1).

3.7. Effect of WF-LPWE on KCl-induced contraction

Incubation of aortic rings with the tissue bath concentration of WF-LPWE at 2.0 mg/mL evoked a significant reduction of KCl at 30–90 mM (p < 0.05) (unpublished data).

3.8. Effect of WF-LPWE on intracellular and extracellular calcium release

To assess the role of intracellular calcium mobilization, the aortic rings were incubated with WF-LPWE in Ca2+-free medium containing 0.1 mM EGTA. PE induced contraction responses in denuded aortic rings in Ca2+-free medium. Aortic rings were incubated with and without 0.5 mg WF-LPWE, produced contraction 1 and 2, respectively. The ratio of the two contractions showed that the contractile response to PE were significantly reduced (p < 0.01) in the present of WF-LPWE
Fig. 1. Concentration-relaxation response curve of various extracts (A) and fractions (B) of *Labisia pumila* and concentration-relaxation response curves of *Labisia pumila* on PE-precontracted endothelium-intact (C) and endothelium-denuded (D) aortic rings. Comparison between groups for (A) and (B) was performed by two-way ANOVA followed by Bonferroni post hoc test and comparison between groups for (C) and (D) was performed by Student’s t-test. Values are expressed as mean ± S.E.M. for six aortic rings (n = 6). *p < 0.1 **p < 0.01 ***p < 0.001.

Table 1
Tabular summary of the maximum relaxation (Rmax) and EC50 value of various type of *Labisia pumila* crude extracts and fractions; and the vasorelaxant effect of WF-LPWE on aortic rings preincubated with different inhibitors. The results are presented as the best-fit values of means ± S.E.M. for six aortic rings (n = 6). Data were analysed using Student’s t-test and two-way ANOVA followed by Bonferroni’s post hoc test.

| Maximum relaxation (Rmax) and EC50 value of various type of *Labisia pumila* crude extracts and fractions; and the vasorelaxant effect of WF-LPWE on aortic rings preincubated with different inhibitors |
|---------------------------------|-----------------|-----------------|-----------------|
| **Group**                       | **Crude Extracts of *L. pumila*** | **Fractions of LPWE** | **Denuded** |
| **n**                           | **Rmax (% relaxation)** | **EC50 (-log EC50)** | **Rmax (% relaxation)** | **EC50 (-log EC50)** | **Rmax (% relaxation)** | **EC50 (-log EC50)** |
| Control                         | 2.86 ± 4.5       | 1.6 ± 0.1       | 5.7 ± 1.0       | 0.2 ± 0.6       | 70.4 ± 7.6          | 0.6 ± 0.0       |
| LPWE                            | 161.0 ± 16.3*    | 0.2 ± 0.1       | 174.3 ± 1.7*    | 0.6 ± 0.0       | 80.0 ± 13.3         | 0.5 ± 0.2       |
| LPPE                             | 125.1 ± 10.1*    | 1.0 ± 0.5       | 70.4 ± 7.6      | 0.6 ± 0.0       | 93.7 ± 5.6          | 0.7 ± 0.1       |
| LPIMEOH                         | 170.3 ± 6.5*     | 0.4 ± 0.3       | 81.6 ± 5.5      | 0.4 ± 0.1       | 98.4 ± 10.9         | 1.7 ± 0.1       |
| LPMEA                           | 136.8 ± 22.0*    | 0.6 ± 0.5       | 136.8 ± 3.1*    | 0.7 ± 0.1       | 122.6 ± 14.8*       | 1.1 ± 0.5       |
| LPCHLOR                         | 106.5 ± 4.1*     | 0.5 ± 0.2       | 106.5 ± 4.1*    | 0.5 ± 0.2       | 119.8 ± 5.2*        | 0.4 ± 0.3       |
| LPET                            | 3.6 ± 4.7        | 0.8 ± 4.0       | 3.6 ± 4.7       | 0.8 ± 4.0       | 3.6 ± 4.7           | 0.8 ± 4.0       |
| LP-LPWE                         | 113.8 ± 3.1*     | 0.7 ± 0.1       | 113.8 ± 3.1*    | 0.7 ± 0.1       | 113.8 ± 3.1*        | 0.7 ± 0.1       |
| LP-LPWE + L-NAME                | 122.6 ± 14.8*    | 1.1 ± 0.5       | 122.6 ± 14.8*   | 1.1 ± 0.5       | 122.6 ± 14.8*       | 1.1 ± 0.5       |
| LP-LPWE + EAF-LPWE              | 113.8 ± 3.1*     | 0.7 ± 0.1       | 119.8 ± 5.2*    | 0.4 ± 0.3       | 113.8 ± 3.1*        | 0.7 ± 0.1       |
| LP-LPWE + BF-LPWE               | 119.8 ± 5.2*     | 0.4 ± 0.3       | 119.8 ± 5.2*    | 0.4 ± 0.3       | 119.8 ± 5.2*        | 0.4 ± 0.3       |
| LP-LPWE + ODQ                   | 5.7 ± 1.0        | 0.2 ± 0.0       | 174.3 ± 1.7*    | 0.6 ± 0.0       | 80.0 ± 13.3         | 0.5 ± 0.2       |
| LP-LPWE + Indomethacin          | 6.0 ± 0.0        | 0.6 ± 0.0       | 70.4 ± 7.6      | 0.6 ± 0.0       | 93.7 ± 5.6          | 0.7 ± 0.1       |
| LP-LPWE + Methylene Blue        | 81.6 ± 5.5       | 0.4 ± 0.1       | 81.6 ± 5.5      | 0.4 ± 0.1       | 98.4 ± 10.9         | 1.7 ± 0.1       |
| LP-LPWE + ODQ + L-NAME          | 87.5 ± 6.7       | 3.2 ± 0.2       | 87.5 ± 6.7      | 3.2 ± 0.2       | 87.5 ± 6.7          | 3.2 ± 0.2       |
| LP-LPWE + ODQ + Indomethacin    | 50.3 ± 4.4*      | 0.6 ± 0.2       | 50.3 ± 4.4*     | 0.6 ± 0.2       | 50.3 ± 4.4*         | 0.6 ± 0.2       |
| LP-LPWE + ODQ + Methylene Blue  | 42.0 ± 8.8       | 0.3 ± 0.5       | 42.0 ± 8.8      | 0.3 ± 0.5       | 42.0 ± 8.8          | 0.3 ± 0.5       |
| LP-LPWE + ODQ + Indomethacin    | 79.1 ± 7.8       | 0.1 ± 0.3       | 79.1 ± 7.8      | 0.1 ± 0.3       | 79.1 ± 7.8          | 0.1 ± 0.3       |
| LP-LPWE + Atropine              | 53.6 ± 4.6       | 1.6 ± 0.2       | 53.6 ± 4.6      | 1.6 ± 0.2       | 53.6 ± 4.6          | 1.6 ± 0.2       |
| LP-LPWE + Prazosin              | 39.7 ± 10.0      | 1.9 ± 0.3       | 39.7 ± 10.0     | 1.9 ± 0.3       | 39.7 ± 10.0         | 1.9 ± 0.3       |
| LP-LPWE + Prazosin              | 62.9 ± 2.3       | 0.3 ± 0.1       | 62.9 ± 2.3      | 0.3 ± 0.1       | 62.9 ± 2.3          | 0.3 ± 0.1       |
| LP-LPWE + Propranolol           | 65.7 ± 3.3       | 1.0 ± 0.2       | 65.7 ± 3.3      | 1.0 ± 0.2       | 65.7 ± 3.3          | 1.0 ± 0.2       |

*p < 0.001 vs. control.*p < 0.001 vs. WF-LPWE before inhibitor.
(0.5) as compared to the absence of WF-LPWE (1.1), as shown in Fig. 2C.

Cumulative addition of CaCl₂ (0.01–10 mM) on aortic rings in Ca²⁺-free Kreb’s solution treated with 0.5 mg/mL WF-LPWE showed a significant rightward shift in the contraction-curve response as compared with control. The contraction was significantly reduced \( (p < 0.01) \) in the presence of WF-LPWE (0.5 mg/mL), as shown in Fig. 2D.

3.9. Identification of gallic acid and catechin in WF-LPWE

A simultaneous determination by reverse-phase HPLC showed good separation of the two biomarker compounds (gallic acid and catechin) in the WF-LPWE as shown in Fig. 3. Gallic acid and catechin in WF-LPWE were then quantified by applying the regression equations of the reference compounds. The results were presented as average w/w. The WF-LPWE extracts were found to contain gallic acid and catechin of 0.33 and 2.71 μg/mL, respectively.

4. Discussion

Our preliminary study found that L. pumila produced vasorelaxant effects, thus this plant merits to be investigated for its vascular activities particularly on the vasorelaxant effects.

LPWE had exhibited the most potent vasorelaxant effect. Thus, LPWE had been further fractionated to ethyl acetate fraction (EAF-LPWE), n-butanol fraction (BF-LPWE) and the residual water fraction (WF-LPWE). We established that WF-LPWE had produced the best significant vasorelaxant effects and reduction of PE-induced contraction on isolated aortic rings of spontaneously hypertensive rats, and thus was further investigated for the mechanistic study.

Activation of endothelial nitric oxide synthase (eNOS) increases the production of NO, the most potent vasodilator [8] and causes vasorelaxation. In order to assess the contribution of NO released in the vasorelaxant effects elicited by L. pumila, we compared the vasorelaxant effect of WF-LPWE on aortic rings pre-contracted with PE (1 μM) with and without L-NAME (10 μM), a NO synthase inhibitor. The result showed that L-NAME did not significantly influence the vasorelaxant effect of the fraction, suggesting that eNOS did not involve in the vasorelaxant effect of WF-LPWE.

NO-induced relaxation in the smooth muscle of blood vessel is mediated via the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway [9]. Since our finding indicated that NO did not play a role in the relaxation induced by WF-LPWE, the involvement of NO-independent induced relaxation of sGC/cyclic GMP pathway was investigated. Aortic rings were incubated with methylene blue and there was no significant inhibitory effect on WF-LPWE induced-relaxation had been observed. Since methylene blue is a non-selective inhibitor of NO-sGC pathway of vascular relaxation [10], we explored further by using 1H- [1,2,4]oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), a selective inhibitor of sGC, in order to investigate the NO-independent direct involvement of sGC/cyclic GMP...
pathway in the vasorelaxation activity of WF-LPWE. ODQ is more potent and specific inhibitor of sGC than methylene blue. Thus, ODQ is an important tool in differentiating between cyclic GMP-dependent and -independent effects of NO [10]. The relaxation effect of WF-LPWE had been significantly inhibited by ODQ indicating the involvement of the NO-independent sGC/cyclic GMP pathway in WF-LPWE-induced relaxation. Our result agrees with Bello et al. (2015) that ODQ significantly diminished the relaxation effect of his studied plant suggesting a direct activation of the cGMP-protein kinase G (PKG) pathway which in turn reduces myosin light chain kinase (MLCK) activities [11].

Prostacyclin (PGI2) plays an important role in endothelium-derived relaxing factor (EDRF). To study the involvement of PGI2 in the relaxation effect of WF-LPWE, aortic rings were preincubated with indomethacin, a nonselective cyclooxygenase (COX) inhibitor. Our results showed that indomethacin did not attenuate the vasorelaxant effect of WF-LPWE suggesting that PGI2 has no role in the relaxation mechanism of WF-LPWE.

Vasoconstriction of vascular smooth muscle is initiated by an increase of intracellular calcium level, achieved via the receptor-operating calcium channel (ROCC) evoked by agonists such as phenylephrine (PE) and/or release of intracellular Ca2+ from intracellular stores via inositol 1,4,5-triphosphate (IP3) receptors [12]. When calcium influx are inhibited, relaxation occurs due to Ca2+ concentration decreases, resulting in inactivation of MLCK and dephosphorylation of the myosin light chain (MLC) by myosin light chain phosphatase (MLCP) [13,14]. In order to assess the role of intracellular calcium mobilization, the aortic rings were incubated with WF-LPWE in Ca2+-free medium containing 0.1 mM EGTA. Under this condition, PE-induced transient contraction mainly using Ca2+ from the sarcoplasmic reticulum. WF-LPWE (0.5 mg/mL) significantly suppressed the contraction induced by PE suggesting that the vasorelaxation exerted by WF-LPWE may possibly due to inhibition of intracellular Ca2+ release from the sarcoplasmic reticulum. Increase the cGMP formation inhibits Ca2+ entry into the cell, thus causes decreases intracellular Ca2+ concentrations, activates potassium channels which leads to hyperpolarization and stimulates PKG that activates MLCP, which all lead to smooth muscle relaxation [15]. Decreased intracellular Ca2+ concentration and increased MLCP activity caused the smooth muscle to undergo weaker vascular contractility. This could probably explain our result which showed that PE-induced phasic contraction of aortic rings in Ca2+-free medium was drastically suppressed after incubation with WF-LPWE.

To evaluate the effect of WF-LPWE on calcium channels on smooth muscle cells, WF-LPWE (0.5 mg/mL) was incubated with aortic tissue in calcium free Kreb’s solution and calcium was added to the organ bath chamber. The addition of CaCl2 (0.1–10 mM) in a concentration-dependent manner in the control group resulted into a gradual increase in the contraction of aorta. However, the contraction of aortic ring was inhibited in the chamber where the aortic ring was incubated with WF-LPWE. This finding supported the inhibitory effect of WF-LPWE on ROCC channels that halted the tissue contraction induced by Ca2+ influx of extracellular calcium thus suggested that calcium channel blockade of ROCC involved in the vasorelaxation process by WF-LPWE.

Potassium ion (K+) channels regulate the membrane potential in smooth muscle cells. Efflux of K+ due to the opening of K+ channels in vascular smooth muscle cause membrane hyperpolarization [16]. The closure of voltage-gated calcium channel (VDCC) leads to a reduction in extracellular Ca2+ entry and cause vasorelaxation [17]. Our present study showed that, at 2.0 mg of WF-LPWE significantly inhibited the contraction induced by potassium chloride (KCl) at 30–90 mM, suggesting that the opening of K+ channels involved in the vasorelaxant effects only at
higher dose of WF-LPWE. Furthermore, the vasorelaxant effect of WF-LPWE did not significantly inhibited by glibenclamide, a non-specific ATP-sensitive K\(^+\) channel blocker. Therefore, our finding suggested that the vasorelaxant effect of WF-LPWE was much lesser contributed by VDCC rather the ROCC.

Both, \(\alpha\)-adrenergic and \(\beta\)-adrenergic antagonist, prazosin and pranolol, were respectively found to have no significant influence on the WF-LPWE induced relaxation, suggesting that adrenergic receptors-blockade is not a factor in the reduction of PE-mediated contraction by WF-LPWE.

\(L.\ pumila\) contains several bioactive compounds that contribute to the pharmacological properties associated with cardiovascular activities. Previous study by Yusoff and Mohamud, (2011) reported that gallic acid, a well-known antioxidant had been identified in the water extract of \(L.\ pumila\) var. \(alata\) [7]. Furthermore, study had demonstrated that gallic acid possessed both, endothelium-dependent vasorelaxant and antihypertensive activities. Gallic acid may have a synergic therapeutic effect on vascular diseases associated with endothelial dysfunction and hypertension [18]. In our study, reverse-phase HPLC was used to identify the biomarker compounds that may contribute to vasorelaxant effect of WF-LPWE and revealed the presence of two biomarker compounds; there were gallic acid and catechin. Thus, we believed that the vasorelaxant effect exhibited by WF-LPWE in our study was contributed of these two biomarker compounds. Recently, Jin et al. (2017) suggested that gallic acid may have promising therapeutic effect on cardiovascular diseases, such as hypertension and cardiac hypertrophy based on their study that gallic acid potentially lowered systolic blood pressure in rats with essential hypertension through inhibition of vascular contractility and reducing aortic wall thickness [19].

5. Conclusion

The inhibitory effects of WF-LPWE on the contractions induced by Ca\(^{2+}\) release from the intracellular sarcoplasmic store and the Ca\(^{2+}\) influx of extracellular calcium suggests a calcium channel blockade of ROCC involved in the vasorelaxation process. Therefore, WF-LPWE exerts its vasorelaxant effect possibly by inhibiting the release of calcium from intracellular calcium stores, antagonising the receptor-operated calcium channels, and inhibiting formation of inositol 1,4,5-triphosphate. WF-LPWE vasorelaxant effect is mediated via NO-independent direct involvement of sGC/cyclic GMP pathway. Gallic acid and catechin might have been playing important roles for the vasorelaxation activities of WF-LPWE.

Conflicts of interest

We wish to inform that there is no known conflict of interest associated with this publication.

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