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Vasorelaxant Properties of *Loranthus ferrugineus* Roxb. Methanolic Extract

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Abstract: In the present study, *Loranthus ferrugineus* was evaluated for blood pressure lowering activity using *in vitro* and *in vivo* animal experimental approaches. The fresh aerial parts of the plant were dried, pulverized into powder and successively extracted with petroleum ether, chloroform, ethyl acetate, methanol and water using hot extraction methods. Each of the extracts was dried under reduced pressure using rotary evaporator and subsequently freeze-dried. The effects of three different concentrations (0.5, 1 and 2 mg mL⁻¹ of each extract were examined on isolated rat aortic ring preparations and responses to cumulative doses of noradrenaline (NA) were measured. It was found that the relatively polar methanol extract was the most potent to produce a significant (p<0.05) dose-dependent inhibition in the maximum response and to shift the dose-response curve of NA to the right which suggests that *Loranthus ferrugineus* methanolic extract (LFME) contains compound(s) with non-competitive inhibitory activity. In another set of experiments, LFME was found to be the most active in blood pressure lowering activity in anesthetized normotensive Sprague Dawley (SD) rat model. Moreover, LFME produced a dose-dependent blood pressure lowering effect. Chemical analysis of LFME showed the presence of significant amounts of polyphenolic and flavonoid constituents. The data suggests that LFME contains some biologically active substances that produce a significant dose-dependent blood pressure lowering effect and vasodilatation is one of the possible mechanisms which may explain its use in the management of hypertension. Furthermore, LFME effects can possibly be attributed to the high polyphenolic contents of this plant.

Key words: Hypertension, *Loranthus ferrugineus* Roxb., aortic rings, noradrenaline, Sprague Dawley rat

INTRODUCTION

Loranthus ferrugineus Roxb. (syn.: *Scurrula ferruginea*) from the *Loranthaceae* family is a hemiparasitic shrub that grows on dicotyledonous trees and attaches itself to the host tree by modified roots (Barlow, 1991; Bengal, 1987). The members of the *Loranthaceae* (about 74 genera), which are generally known as mistletoes and locally known as dedalu or dalu-dalu, are widely distributed in many tropical countries like Malaysia, Sumatra and India. In Malaysia, preparations from *L. ferrugineus* have been widely used in traditional medicine for several therapeutic purposes. As a reputable home remedy, beside its use for treatment of hypertension and gastrointestinal complaints, decoction of *L. ferrugineus* is also taken for general health and for its gerontological effect. Leaves, fruits and flowers are the

most common parts of *L. ferrugineus* used to treat high blood pressure, while the roots attaching it to the host plant are meant for other therapeutic uses such as ulcer and cancer treatment (Jaafar, 1985).

While herbs have been a major source of many pharmaceutical preparations and are often approved as nonprescription medications in many countries around the world (i.e., both industrialized and developing countries), the range of clinical applications, in addition to uses in self-medication, for herbs are tremendously wide (Blumenthal, 2003).

Prominent examples of prospective clinical applications of herbs and phytomedicines, where the herbs play either a primary or adjunct role, are their implication in increased peripheral circulation, for example in peripheral arterial occlusive disease (intermittent claudication) and Raynaud's disease; Congestive Heart

Failure (CHF) stages 1 and 2 (increased central circulation and positive inotropic activity of heart); hyperlipidemia, atherosclerosis and hypertension (Blumenthal, 2003).

We report in this investigation the vascular and blood pressure lowering effects of different extracts obtained from *L. ferrugineus* using *in vitro* and *in vivo* experimental approaches. *In vitro* protocol included the utilization of SD rat aortic rings preparations while *in vivo* protocol encompassed the use of blood pressure recording in anesthetized SD rat animal model.

MATERIALS AND METHODS

Plant materials: Fresh aerial parts (leaves, stems, twigs, flowers and berries) of *L. ferrugineus* (10 kg) were collected from one of the botanical gardens in the main campus of Universiti Sains Malaysia (USM), Penang, Malaysia in July 2007 and authenticated by Mr. Adnan Bin Jaafar, a taxonomist in the School of Biological Sciences, USM. A voucher specimen (No. 10943) is kept in the herbarium of the same institute.

Preparation of crude extract: The plant material was cleaned of adulterants and dried in the oven at 42°C for 5 days. The dried plant material was ground into a fine powder using a milling machine. By means of hot extraction methods, the powdered plant material (400 g) was packed into a Soxhlet thimble and successively extracted with petroleum ether, chloroform, ethyl acetate and methanol using the Soxhlet apparatus (Schott, Duran, Germany). On the other hand, water extract was obtained using a reflux apparatus (Schott, Duran, Germany). These extracts were then concentrated on a rotary evaporator (Büchi, Switzerland) under reduced pressure (-760 mmHg) and subsequently freeze-dried. The plant's yields obtained for each extract were as follows: petroleum ether extract, a dark brown wax-like material, was 0.9%; chloroform extract, a hard black material, was 0.3%; ethyl acetate extract, a brown waxy material, was 1.4%; methanol extract, a dark brown semi-solid material, was 11.8% and finally, water extract, a reddish-brown semi-solid material, was 2.8%.

Drugs and solutions: For extraction process, petroleum ether, chloroform, ethyl acetate and methanol were purchased from Fisher Scientific, UK. For *in vitro* experiments, NA was purchased from Sigma-Aldrich, Germany while NaCl, KCl, CaCl₂·2H₂O, MgSO₄, KH₂PO₄, glucose, NaHCO₃ and EDTA were purchased from R and M Chem., UK. Pentobarbitone sodium (Rhône Merieux) and heparin (Leo Pharmaceuticals) for *in vivo* experiments were used as commercially available injectable solutions.

For experimental determination of total phenolic contents and total flavonoids contents, Folin-Ciocalteu reagent, gallic acid and quercetin were purchased from Sigma-Aldrich, Germany while sodium carbonate, aluminum chloride and potassium acetate were bought from R and M Chem., UK.

For *in vitro* protocol and just before use, LFME and NA were immediately dissolved in Kreb's physiological solution of following composition (in mM): NaCl 118.2, KCl 4.7, CaCl₂·2H₂O 2.5, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11.7, NaHCO₃ 25.0 and EDTA 0.026 while petroleum ether, chloroform and ethyl acetate extracts were dissolved using 0.5% PEG400 in Kreb's solution.

For *in vivo* protocol, petroleum ether, chloroform and ethyl acetate extracts were solubilized using 0.5% PEG400 (Sigma-Aldrich, Germany) in isotonic normal saline followed by sonication. LFME and water extract were dissolved in normal saline (0.9% sodium chloride) just before use. All i.v. boluses were administered in a volume of 200 µL. Each i.v. dose was followed by 100 µL flush of saline to ensure complete delivery of the dosage.

Animals: Experimental animals consisted of adult male SD rats 250-300 g which were bred and obtained from USM Animal House Facility, Penang, Malaysia. The animals were housed in standard environmental conditions (25°C, 60-70% humidity) under natural lighting and fed with normal commercial rat chow (Gold Coin Feed mills Sdn Bhd, Malaysia) and water *ad libitum*. They also were allowed to acclimatize in the animal transit room for a minimum period of 1 week before being used for any experiment. Animals handling and all procedures on animals were carried out in accordance with the guidelines of the Animal Ethics Committee, USM, Penang, Malaysia and had their approval. The number of determinations for each *in vitro* isolated rat aortic rings experiment was 8 while each experimental group consisted of 6 animals for the *in vivo* experiments.

Preparation of rat thoracic aorta rings and experimental protocol: The rats were anesthetized with pentobarbitone sodium (60 mg kg⁻¹ of body weight, i.p.) and subsequently exsanguinated. The thoracic aorta was carefully isolated, cleaned of fat and connective adipose tissue and cut into 3-5 mm long rings. The rings were suspended horizontally in tissue chambers containing 10 mL of Kreb's physiological solution. Special care was taken to avoid damage to the endothelium. The tissue-bath solution was aerated incessantly with 95% O₂ and 5% CO₂ at 37 °C. Aortic rings were allowed to equilibrate at an optimal tension of 1 g for 30 min. During this period,

Kreb's solution was replaced every 15 min and, if needed, the tension was readjusted to 1 g. Before starting actual experimental protocol, contractile responsiveness of the aortic rings was tested by repeated exposure to NA solution to obtain final bath concentrations of $1.0\text{E-}10$, $3.0\text{E-}10$, $1.0\text{E-}9$, $3.0\text{E-}9$, $1.0\text{E-}8$, $3.0\text{E-}8$, $1.0\text{E-}7$, $3.0\text{E-}7$, $1.0\text{E-}6$, $3.0\text{E-}6$, $1.0\text{E-}5$ and $3.0\text{E-}5\text{M}$. Responses were recorded isometrically via a force-displacement transducer (P23 ID Gould, Statham Instrument, UK) connected to Grass polygraph model 79D (Quincy, Mass., USA) (Ajay *et al.*, 2003).

Successive doses of NA were added to the tissue bath after the tissues had acquired steady state equilibrium. After the maximum response had been achieved, NA was then washed out of the bath and complete relaxation of the tissue was allowed. Three concentrations (0.5 , 1 and 2 mg mL^{-1}) of each extract were prepared by dissolving in Kreb's solution. These concentrations were then added successively to the tissue bath and allowed to equilibrate with the tissue for 20 min. The tissue was then exposed to the earlier mentioned NA doses and dose-response curves were subsequently obtained for all the concentrations of the extracts. In another set of experiments, the concentration of PEG400 used to suspend petroleum ether, chloroform and ethyl acetate extract was proved not to affect the response to NA-induced contraction of aortic rings.

Animal surgical procedure and experimental protocol:

Animals were starved overnight with unlimited access to drinking water. Anesthesia was induced with pentobarbitone sodium at a dose of 60 mg kg^{-1} (i.p.). Blunt incision was carried out to separate the muscles of the neck and 1 cm long of trachea was pulled up. A slanting cut was made on the trachea and a PP240 endotracheal cannula was inserted into the trachea to provide a clear airway passage. The left Jugular Vein (JV) was then cannulated with PP50 tubing to enable administration of additional bolus injections of anesthetic (10 mg kg^{-1} in normal saline i.v.) periodically and to allow the administration of bolus doses of the plant extracts. The right carotid artery was similarly catheterized with PP50 tubing filled with heparinized saline (50 IU of heparin mL^{-1} normal saline) for direct measurement of Mean Arterial Blood Pressure (MAP) via a pressure transducer (P23 ID Gould, Statham Instrument, UK) connected to Grass polygraph model 79D (Quincy, Mass, USA). Thereafter, a small abdominal incision was carried out using an electrical cutlery knife (Model HDCS, Rimmer Brothers) to minimize bleeding and the urinary bladder was pulled up. Subsequently a small cut on the urinary bladder was made and a PP10 cannula was inserted to

allow free passage of urine from the kidneys. Upon completion of surgery, 2 mL of normal saline were given slowly and intravenously through the JV and the animal was allowed to equilibrate and stabilize for 30 min before commencing the administration of plant extracts. Right after stabilization, control values for MAP were obtained and then for the vehicle (PEG400 in some of the experiments, data not shown) or increasing doses of each extract (25 , 50 , 100 and 200 mg kg^{-1} in 10 min intervals) administered as bolus i.v. injections over 15 sec were recorded.

Chemistry: Based on the *in vitro* and *in vivo* experimental results and following determination of the most potent plant extract to exert the presumed pharmacological effect, further attempts were carried out to chemically define the composition of the active crude extract using the chemical analytical procedures below:

Determination of total phenolic contents: The total soluble phenolic contents in the most active plant extract of *L. ferrugineus* were determined by using Folin-Ciocalteu reagent and gallic acid (also known as 3,4,5-trihydroxybenzoic acid) as a standard according to the method of Slinkard and Singleton (Slinkard and Singleton, 1977). A solution of 4 mg mL^{-1} of the active plant extract in methanol and solutions of 0.0625 , 0.125 , 0.25 , 0.5 , 1 , 2 and 4 mg mL^{-1} of gallic acid in methanol were prepared. Twenty microliters of the active plant extract and each concentration of gallic acid solution were pipetted in separate test tubes followed by the addition of 1.58 mL of distilled water and $100\text{ }\mu\text{L}$ of 2 N Folin-Ciocalteu reagent. Subsequently, the test tubes were mixed thoroughly. After 8 min, $300\text{ }\mu\text{L}$ of 20% sodium carbonate solution were added. The mixture was then allowed to stand for 2 h with intermittent shaking. The absorbance of solutions was measured at 765 nm with a Hitachi U-2000 spectrophotometer (Hitachi, Japan).

The concentration of total phenolic compounds in the extract was determined as milligrams of gallic acid equivalent by using an equation which was obtained from a standard gallic acid graph.

Determination of total flavonoid content: The total flavonoid contents in the most active plant extract of *L. ferrugineus* were determined by using aluminum chloride colorimetric method with quercetin as standard (Chang *et al.*, 2002; Kosalec *et al.*, 2004; Woisky and Salatino, 1998). A solution of 4 mg mL^{-1} of the active plant extract in methanol and solutions of 0.0078 , 0.0156 , 0.0313 , 0.0625 , 0.125 , 0.25 , 0.5 , 1 , 2 and 4 mg mL^{-1} of quercetin in methanol were prepared. Five hundred microliters of the active plant extract and each concentration of quercetin

solution were pipetted in separate test tubes followed by the addition of 0.1 mL of 10% (w/v) aluminum chloride solution, 0.1 mL of 1 M potassium acetate solution, 1.5 mL of methanol and 2.8 mL of distilled water. The test tubes were thoroughly mixed and after incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with a Hitachi U-2000 spectrophotometer (Hitachi, Japan). The amount of 10% aluminum chloride was substituted by the same amount of distilled water in a blank. The concentration of total flavonoid contents in the extract was determined as micrograms of quercetin equivalent by using an equation that was obtained from a standard quercetin graph.

Statistical analysis: All data were expressed in terms of Mean \pm SEM. The statistical analysis of the data was done using one- and two-way ANOVA followed by the Bonferroni/Dunnett post hoc test (SuperANOVA, Abacus Concepts, Inc., Berkeley, CA, USA). The differences between the means were considered significant at the 5% level. The median effective concentration (EC₅₀) was analyzed by linear regression.

RESULTS AND DISCUSSION

In vitro experiments

Petroleum ether extract: At a tissue bath concentration of 0.5 mg mL⁻¹ *L. ferrugineus* petroleum ether extract showed a significant enhancement of NA action on rat aortic rings as compared to the control (68.1 \pm 6.3% versus 47.2 \pm 3.9%; $p < 0.05$) which may indicate the presence of some components that augment NA-induced vascular smooth muscle contraction at lower concentrations. Concentrations of 1 and 2 mg mL⁻¹ petroleum ether extract were not significantly different from the control (42.2 \pm 4.6 and 40.6 \pm 4.7%, respectively vs. 47.2 \pm 3.9%; $p > 0.05$) (Fig. 1). The latter finding may signify the presence of some components that come into play at higher concentrations to antagonize the effects of those constituents responsible for the pattern of enhanced NA response on aortic rings at lower concentrations.

Chloroform extract: A tissue bath concentration of 0.5 mg mL⁻¹ *L. ferrugineus* chloroform extract showed a similar action to the same concentration of petroleum ether extract whereby it significantly enhanced NA effect on rat aortic rings when compared to the control (65.1 \pm 6.5% versus 44.2 \pm 4.0%, $p < 0.05$) but unlike petroleum ether extract a concentration of 1 mg mL⁻¹ chloroform extract produced a significant drop in NA-induced aortic rings contraction (35.9 \pm 3.9% versus

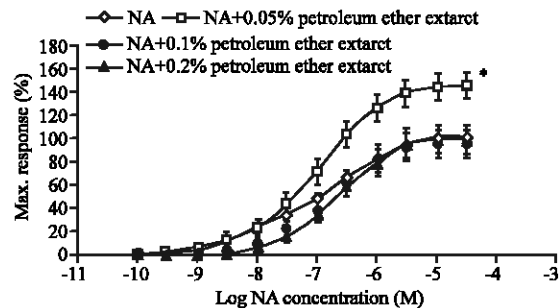


Fig. 1: The effect of *L. ferrugineus* petroleum ether extract on maximum response (%) in noradrenaline (NA)-induced vascular smooth muscle contraction in rat aortic rings preparations. Data presented as Mean \pm SEM (n = 8). * $p < 0.05$, the effect of 0.5 mg mL⁻¹ *L. ferrugineus* petroleum ether extract on NA-induced aortic rings contraction versus control. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

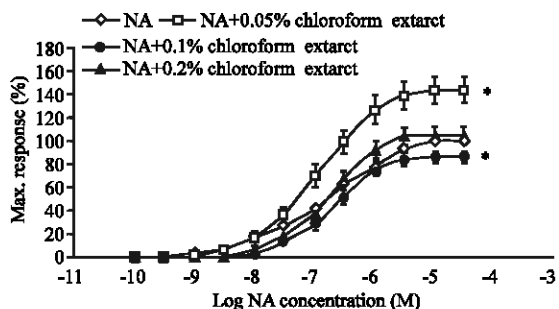


Fig. 2: The effect of *L. ferrugineus* chloroform extract on maximum response (%) in noradrenaline (NA)-induced vascular smooth muscle contraction in rat aortic rings preparations. Data presented as Mean \pm SEM (n = 8). * $p < 0.05$, the effects of 0.5 and 1 mg mL⁻¹ *L. ferrugineus* chloroform extract on NA-induced aortic rings contraction versus control. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

44.2 \pm 4.0%, $p < 0.05$). Higher concentrations of chloroform extract did not show any significant effect on the aortic rings as compared to the control (44.9 \pm 4.8% versus 44.2 \pm 4.0%, $p > 0.05$). These findings may indicate that chloroform extract contains contractile-response-enhancing and vasorelaxing elements, however their diverse actions appear to be concentration-dependent (Fig. 2).

Ethyl acetate extract: Tissue bath concentrations (0.5, 1 and 2 mg mL⁻¹) of *L. ferrugineus* ethyl acetate extract showed significant dose-independent reduction in NA-induced vasoconstriction as compared to the control

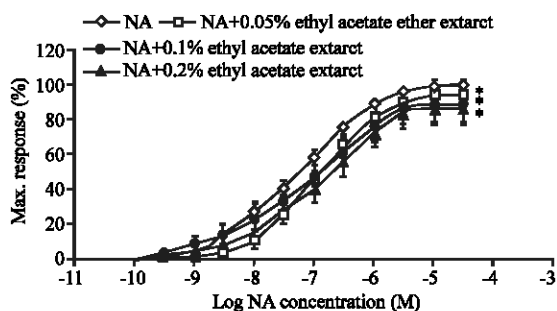


Fig. 3: The effect of *L. ferrugineus* ethyl acetate extract on maximum response (%) in noradrenaline (NA)-induced vascular smooth muscle contraction in rat aortic rings preparations. Data presented as Mean±SEM (n = 8). *p<0.05, the effects of 0.5, 1 and 2 mg mL⁻¹ *L. ferrugineus* ethyl acetate extract on NA-induced aortic rings contraction versus control. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

(42.9±4.2, 44.3±4.0 and 40.6±4.0%, respectively versus 50.5±4.0%; p<0.05), indicating the presence of some vasorelaxing elements in this crude extract whose actions lack the ability to dose-dependently relax blood vessel by gradual geometrical increase in tissue bath concentration (Fig. 3).

Methanol extract: Concentrations of 0.5, 1 and 2 mg mL⁻¹ LFME produced a highly significant and dose-dependent reduction in NA-induced contraction of isolated rat aortic rings preparations as compared to the control (29.6±3.2, 30.4±3.3 and 33.3±3.8%, respectively versus 48.1±4.1%; p<0.05) with EC₅₀ values of 6.8E-7M, 6.2E-7M and 4.9E-7M respectively as compared to the control one (9.0E-8M). Additionally, it shifted the dose-response curve to the right, which indicates the presence of high concentration of promising vasorelaxing components in the methanol extract of *L. ferrugineus* (Fig. 4).

Water extract: Similar to petroleum ether extract, *L. ferrugineus* water extract at a tissue bath concentration of 0.5 mg mL⁻¹ showed a significant enhancement of NA action on rat aortic rings (43.7±4.4% versus 51.8±3.9%, p<0.05) which may signify the presence of some components that augment NA effect on vascular smooth muscle and that come into play at lower concentrations. Higher concentrations of 1 and 2 mg mL⁻¹ water extract, on the other hand, significantly enhanced NA action on the rat aortic rings as compared to the control (65.9±5.9 and 60.8±5.5%, respectively; p<0.05) (Fig. 5). Such pattern of dissimilar responses indicates the presence of both

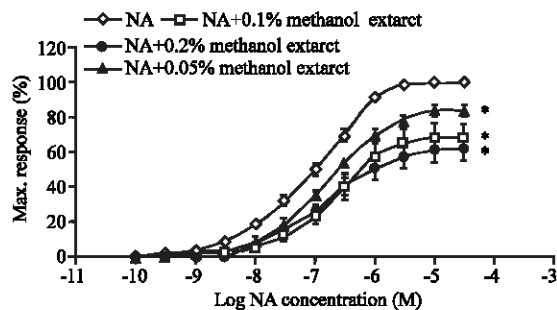


Fig. 4: The effect of *L. ferrugineus* methanol extract on maximum response (%) in noradrenaline (NA)-induced vascular smooth muscle contraction in rat aortic rings preparations. Data presented as Mean±SEM (n = 8). *p<0.05, the effects of 0.5, 1 and 2 mg mL⁻¹ *L. ferrugineus* methanol extract on NA-induced aortic rings contraction versus control. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

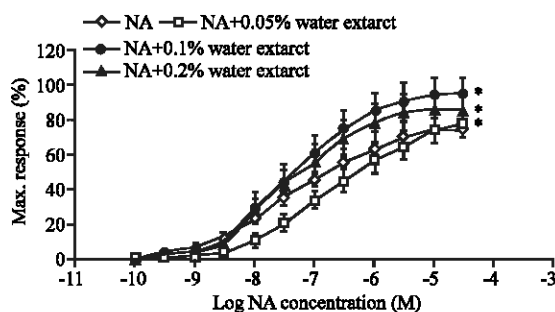


Fig. 5: The effect of *L. ferrugineus* water extract on maximum response (%) in noradrenaline (NA)-induced vascular smooth muscle contraction in rat aortic rings preparations. Data presented as Mean±SEM (n = 8). *p<0.05, the effects of 0.5, 1 and 2 mg mL⁻¹ *L. ferrugineus* water extract on NA-induced aortic rings contraction versus control. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

vasorelaxing and vascular smooth muscle contraction enhancing elements that contribute to vasorelaxation at lower concentrations and profound enhancement of NA effect on isolated rat aorta respectively.

In vivo experiments: Initially and before commencing any extract administration, MAP basal values were obtained and found to be 141±9.3 mmHg. This study showed that the methanolic extract obtained from *L. ferrugineus* possesses significantly (p<0.05) higher capabilities to lower MAP (calculated as % reduction in MAP) as

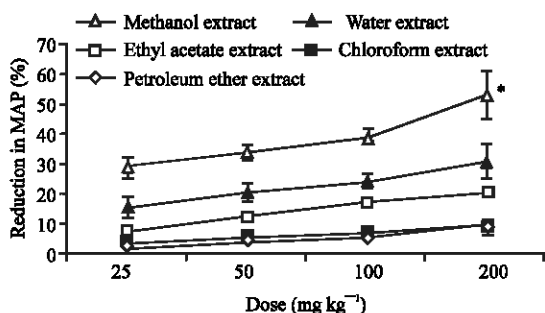


Fig. 6: Percent reduction in mean arterial blood pressure (MAP) elicited by i.v. injection of increasing doses (25-200 mg kg⁻¹) of *L. ferrugineus* petroleum ether, chloroform, ethyl acetate, methanol and water extracts in anesthetized SD rats. Data presented as Mean±SEM (n = 6). *p<0.05, *L. ferrugineus* methanol extract versus petroleum ether, chloroform, ethyl acetate and water extracts. Data were analyzed by two-way ANOVA followed by Bonferonni/Dunnett post-hoc test

compared to the other extracts (methanol extract 38.4±4.2%, water extract 22.7±3.9%, chloroform extract 14.3±2.1%, ethyl acetate extract 6.2±2.0% and petroleum ether extract 5.6±2.0%) (Fig. 6). The significance of this finding lies in the fact that some reports have demonstrated that an aqueous extract obtained from *L. ferrugineus* possesses remarkable capabilities to lower blood pressure *in vivo* (Othman, 1988). The present results contradicted this finding and proved that methanol extract obtained from this plant possesses the most powerful blood pressure lowering properties. This contradiction with previous studies could be attributed to the use of single solvent extraction to obtain an aqueous extract of this plant.

Chemistry: The result of the *in vitro* and *in vivo* experiments suggested that LFME possesses the greatest activity to dose-dependently relax blood vessels and lower blood pressure, forcing the interest to look deeper into the chemical composition of this extract and the possible constituents responsible for these effects. The calculated phenolic contents of LFME were found to be 11.8%, while the total flavonoid contents of the same extract were approximately 1.8%. Flavonoids comprise a large group of naturally existing polyphenolic compounds widely distributed throughout the plant kingdom. These compounds are reported to modulate vascular tone with an ultimate vasorelaxation via endothelial release of nitric oxide and prostaglandins (Amos *et al.*, 2003). The latter

contributes to an essential reduction in the occurrence of cardiovascular diseases.

In summary, the *in vitro* and *in vivo* experiments suggest that LFME is the most active extract among all the extracts obtained from *L. ferrugineus* to produce a dose-dependent drop in NA-induced vascular smooth muscle contraction in isolated SD rat aortic rings preparations and blood pressure in anesthetized SD rat model. LFME possesses the ability to reduce blood pressure in normotensive rats which proves its hypotensive potential. Furthermore, vasodilatation is one of the possible mechanisms. The last finding indicates the presence of biologically active constituents that can be isolated, purified and used for treatment of elevated blood pressure in human. These constituents are most likely of polyphenolic origin as they are well known to possess the ability to reduce blood pressure. Moreover, these findings justify the traditional use of this plant for hypertensive conditions management.

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