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Research Article

Antihypertensive activity of ethanolic extract of *Andrographis paniculata* herbs in wistar rats with a non-invasive method

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ABSTRACT

Hypertension is a main cause of approximately 66% of cardiovascular disease in 15 countries in the Asia Pacific region, including Indonesia. The high cost of treatment and the side effects of drugs are causes of the lack of public awareness in dealing with hypertension. In Indonesia, *Andrographis paniculata* [Burm.f.] Ness grows abundantly, and is widely used as a traditional medicine such as in treatment of hypertension. This study aimed to evaluate the antihypertensive activity of the ethanolic extract of *A. paniculata* herbs (EEAP) in Wistar rats with a non-invasive method. In the study, the herb was extracted by 90% ethanol, and identified for its andrographolide, flavonoids and phenolics contents. Antihypertensive activity was evaluated using CODA "Non Invasive Blood Pressure (NIBP) system with Volume Pressure Recording method". High blood pressure was induced with an alpha adrenergic receptor agonist, phenylephrine (0.9 mg/kgBW). EEAP at doses of 45, 90 and 180 mg/kgBW was evaluated for its antihypertension in comparison to nifedipine (10.8 mg/kgBW). Blood pressure measurements were performed 3 times i.e. before induced (baseline), 15 and 45 minutes after phenylephrine administration. Based on TLC-densitometric data, andrographolide, total flavonoids and total phenolics contents in EEAP were 12.85 ± 0.46%, 0.72 ± 0.01% and 1.66 ± 0.01%, respectively. EEAP exhibited a potent antihypertensive activity in phenylephrine-induced hypertensive rats. EEAP could decrease systolic and diastolic blood pressures up to 120% and 150%, respectively. EEAP fractions are potential to develop as a hypotensive agent in hypertension therapy.

Keywords: *Andrographis paniculata*, antihypertensive, andrographolide, non-invasive method

INTRODUCTION

Heart or cardiovascular disease is the biggest killer in Indonesia and the world[1]. Approximately 66% of cardiovascular disease in 15 countries in the Asia Pacific region including Indonesia is related to hypertension. The incidence of hypertension around the world and approximately 7.1 million out of 1 billion dead is due to hypertension[2]. The prevalence of hypertension ranges 5-47% and 7-38% in men and women, respectively[3]. In early stages, hypertension has no symptoms, so that it will be detected when its complications and clinical symptoms arise[4]. The complications associated with hypertension may be heart attack, heart failure, stroke and kidney diseases[5]. In Indonesia, 75.8 % of hypertension cases have not been diagnosed well by health care system[6]. Some people argued that high cost of treatment and the side effects of synthetic drugs are causes of the lack of public awareness in dealing with hypertension. Based on this fact, traditional medicine can be an alternative option.

Traditional medicine has been widely accepted almost all over the world. WHO fully supports the development and use of traditional medicine. Some countries in Africa, Asia and Latin America have used the herbal medicine as a complementary primary treatment. Even in Africa, as many as 80% of the population has used the herbal medicine for the primary treatment[2]. Many people believe that traditional medicine is relatively safe and no or low side effect in hypertension treatment, however, this statement is still debatable.

A. paniculata with the main content of andrographolide has been widely reported, among other as an antipiretic, anti-inflammatory, anti-allergy, anti-pletelet aggregation, anti-virus, anti-HIV, anti-thrombosis, antidiabetic, immunostimulatory, hepatoprotective and anti-cancer[7-18]. According to Prakash and Manavalan, toxic dose of andrographolide in oral administration was more than 2000 mg/kgBW[19]. The low toxic effect of *A. paniculata* means that the plant is relative safe for traditional use. *A. paniculata* has also been studied for its combination with other plants, formulation, phytochemical, bioavailability studies etc (20-24). In Indonesia, *A. paniculata* grows abundantly and its market price is very cheap. However, the information of its antihypertensive activity is still very limited. Due to lack of scientific information of antihypertensive activity of *A. paniculata*, the study aimed

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to determine the antihypertensive activity of EEAP with a non-invasive method.

MATERIALS AND METHODS

Materials

A. paniculata was collected from area around Jitardukuh Sumberarum Moyudan Sleman Yogyakarta during June 2014. The herb was identified by a botanist at the Department of Pharmaceutical Biology, Universitas Gadjah Mada, and the specimen was deposited in herbarium of the department. Other materials were ethanol 96% (PT Brataco), phenylephrine (Sigma-aldrich.Co), andrographolide (Sigma-aldrich.Co), rutin (Sigma-aldrich.Co), nifedipine (PT Bayer Indonesia), CMC Na (PT Brataco), aqua pro injection (PT Otsuka Indonesia).

Animals

Male Wistar rats aging 2-3 months weighing 200-300 g were obtained from Experimental Animal Development Unit, Laboratory of Pharmacology and Toxicology, Universitas Gadjah Mada, Yogyakarta, Indonesia. The animals were placed in a constant temperature (22 ± 2°C) with a constant relative humidity (55 ± 10%). They were treated in a standard laboratory conditions, cycle dark / light 12 hours and had free access to food and water. This animal handling protocol was in accordance with the guidelines for laboratory animal care Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia. The animal handling protocol was performed in accordance with the previous study[25]. That study was approved by Research Ethics Committee, Integrated Research and Testing Laboratory Universitas Gadjah Mada, Indonesia.

Preparation of EEAP

EEAP was provided by maceration of 0.5 kg dried powder of *A. paniculata* herbs with 90% ethanol at a ratio of 1:5 for 2 days, and with stirring occasionally. Then, the filtrate was collected and the sediment was re-extracted using 90% ethanol at a ratio of 1:2 for overnight. The re-extraction was done twice. The extract was collected, and evaporated to provide a viscous ethanolic extract.

Qualitative analysis of EEAP

Qualitative analysis was performed with a Thin Layer Chromatography (TLC) using andrographolide and rutin as standards. In andrographolide analysis, silica gel 60 F_{254} was used as a stationary phase. Combination of chloroform and methanol (9:1 v/v) was used as a mobile phase. Spots were observed by UV_{254} and UV_{366} after being sprayed with anisaldehid sulfuric acid reagent and heated at 110°C for 10 minutes. In rutin analysis, silica gel 60 F_{254} was used as a stationary phase, and a combination of ethyl acetate:formic acid:glacial acetic acid:water (100:11:11:27 v/v) was used as a mobile phase. Spots were observed by the UV_{254} and UV_{366} after being sprayed with sitroborat reagent.

Quantitative analysis of EEAP

Quantitative analysis of EEAP included calculation of andrographolide, total flavonoids and phenolics contents. Andrographolide level in EEAP were determined by using a TLC densitometric method[26]. Sample of EEAP and a series of concentrations of standard andrographolide were eluted on TLC plate silica gel 60 F_{254}. Combination of chloroform and methanol (9:1 v/v) was used as a mobile phase. Quantitatively, andrographolide was observed with TLC densitometry at a wavelength of 230 nm.

Total flavonoid content was determined regarding to the procedure of Chang et al. using rutin as a standard[27,28]. EEAP and rutin were dissolved in ethanol (5 mg/mL). Rutin solution was diluted to provide a series of standard solutions for a standard curve. Each solution was added with 1.5 ml methanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1 M potassium acetate and 5 ml aquadest. The absorbance was measured at wavelength of 415 nm. Distilled water with 10% $AlCl_3$ was used as a blank. Total flavonoid content was expressed in gram rutin equivalent (RE) of each 100 grams of EEAP dry weight.

Total phenolic content was determined according to the method of Zou et al. using gallic acid as a standard[28,29]. Gallic acid solution was diluted in ethanol to provide a series of standard solutions for a standard curve. Sample of EEAP (10 mg) was placed in 10 mL flask, and then added with 1.5 mL of Folin-Ciocalteau (1:10), and stand for 3 minutes. Furthermore, this solution was added with 1.2 mL of 7.5% Na_2CO_3, and then added with distilled water. After 47 minutes incubation, the absorbance was measured at wavelength of 750 nm versus a blank consisting of distilled water and Folin-Ciocalteau reagent. Total phenolics content was expressed in gram gallic acid equivalent (GAE) of each 100 grams of EEAP dry weight.

Antihypertensive assay

The animals were adapted to the place by measuring their blood pressure once a day for a week. High blood pressure was induced with a sub-cutaneous injection of phenylephrine (0.9 mg / kgBW). In preliminary study, phenylephrine was evaluated for its effect on the profile of rat blood pressure. The rats were divided into 7 groups i.e. negative control group (vehicle), positive control (nifedipine 10mg/kgBW), EEAP with doses of 45, 90 and 180 mg/kgBW. Two more groups were treated without injection of phenylephrine, but administered with aqua pro injection s.c and vehicle (1% CMC Na) p.o, respectively. The initial blood pressure of the subject was measured. Thirty minutes after drug (nifedipine, EEAP) or vehicle (1% CMC Na) treatments, the subjects were injected with phenylephrine, subcutaneously. Systolic, diastolic and mean arterial blood pressure were observed at the minutes of 15 and 45 after phenylephrine injection.

Percentage of reduction in blood pressure was calculated according to the following formula:

$$[100 - (\frac{\text{changes in blood pressure treatment}}{\text{changes in mean blood pressure negative control}} \text{x} 100)]\% \text{ ..... (1)}$$

Statistical analysis

The data were presented as mean ± the standard error of mean (SEM). The data were analyzed statistically using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. The P-values less than 0.05 were considered significant.

RESULTS

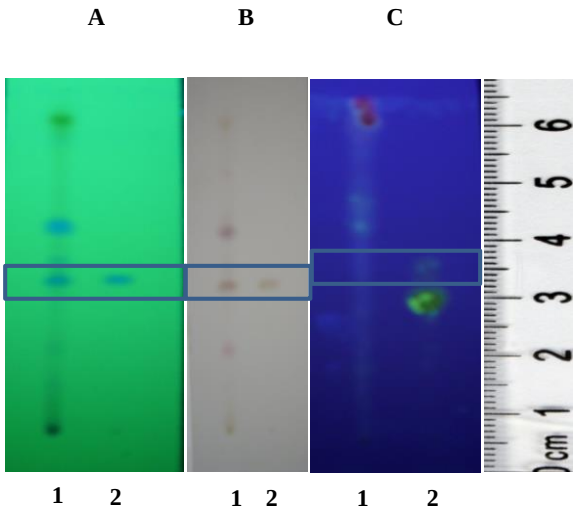

Figure 1. Thin layer chromatography profile of EEAP

Analysis of EEAP

Fig 1a showed the presence of andrographolide in EEAP. Fig 1b showed the content of other terpenes in EEAP. Fig 1c showed the content of flavonoids in the EEAP. Quantitative analysis of EEAP included calculation of andrographolide, total flavonoids and phenolics contents. Based on TLC densitometry, the linear regression equation was Y = 1225 x + 4449 with r = 0.982, and the calculated level of andrographolide was 12.852 ± 0.457%. Analysis of total flavonoids content with the equation Y = 0.030 x - 0.11 and r = 0.995 yielded the content of this compound was 0.715 ± 0.001% RE. Analysis of total phenolics content resulted in a equation Y = 0.0101 x - 0.00131 with r = 0.999 was 1.663 ± 0.008% GAE.

Effect of phenylephrine on the rat blood pressure

In preliminary study, phenylephrine was evaluated for its effect on the profile of rat blood pressure (diastolic, systolic, mean arterial blood pressure). After phenylephrine induction, significant increase of blood pressure occurred at minute 15 and the blood pressure returned into basal value at minute 55 (fig 2). This fact indicates that the duration of action of phenylephrine was 40 minutes. Based on rat blood pressure profile after phenylephrine induction, rat blood pressure measurements in the study were performed twice, at 15 and 45 minutes after induction.

Effect of EEAP on phenylephrine-induced blood pressure

In the preliminary step, subcutaneous treatment of aqua pro injection and peroral treatment of 1% CMC Na p.o. in the absence of phenylephrine induction did not influence the systolic, diastolic and mean arterial blood pressure significantly (table 1). It indicates that the vehicle did not have any effect on the rat blood pressure. In the evaluation of antihypertensive activity of EEAP, three doses of EEAP i.e. 45, 90 and 180 mg/kgBW (suspended in 1 % CMC Na) were tested. Determination of initial blood pressure (at minute 0) and blood pressure at 15 and 45 minutes after induction with phenylephrine were shown in fig 3.

There is no significant difference in terms of percentage of decrease in blood pressure among the EEAP, dose 45 and 90 mg/kgBW, and positive control (nifedipine 10mg/kgBW). However, there was a significant difference between the positive control with EEAP dose of 180 mg/kgBW in minute 15 (table 2). This study had demonstrated that the effects of the EEAP succeeded to reduce systolic, diastolic and mean arterial blood pressure in phenylephrine-induced rats. EEAP could decrease the systolic, diastolic and mean arterial blood pressure up to 120%, 150%, and 140%, respectively. The antihypertensive activities of EEAP were dose-dependent.

DISCUSSION

Indonesia has second larger biodiversity in the world after Brazil including medicinal plants. Exploration of the medicinal plants have been done for their pharmacological activities, herbal formulation, phytochemical studies etc. Since the cardiovascular diseases, cancer and diabetes mellitus are the biggest killer in Indonesia, the studies of medicinal plants for the treatment of these diseases are increasing[30-34].

In the study, the results of TLC profile exhibited the presence of andrographolide in EEAP due to the same spot colour and Rf between certain spot in EEAP with a single spot of standard andrographolide. Besides andrographolide, several spots with different Rf also available in EEAP indicates that EEAP contained several types of terpenes. According to Chao dan Lin, the terpenes in *A. paniculata* were andrographolide, 14-deoxyandrographolide, neoandrographolide,14-deoxy-11,12-didehydro-andrographolide, 14-deoxy-14,15-didehydro-andrographolide, andrographanin, isoandrographolide, 14-acetyl-andrographolide dan 19-O-acetyl-anhydro-andrographolide[35]. Spraying with anisaldehide sulfuric acid on terpenes produced purple red or purple colour that can be observed in visible light.

TLC profile on figure 1c also showed the content of flavonoids in the EEAP. The reaction between flavonoid compounds and sitroborat reagent results in a yellow complex with fluoresence at UV_{366}. According to Chao dan Lin, flavonoid compounds in *A. paniculata* were 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2',5'-tetramethoxyflavone, 5-hydroxy-7,8,2',3'-tetramethoxyflavone, 5-hydroxy-7,8,2'-trimethoxyflavone, 7-O-methylwogonin and 2'-methyl ether[35]. Flavonoids are conjugated aromatic compounds and showed strong absorption in the UV. The intensity is depend on the type of flavonoid[36,37]. Phenolic compounds consist of several structures including the simple monocyclic phenols, phenylpropanoid, phenolic quinines, tannins and flavonoids[36].

In the study, the level of andrographolide in the extract is still lower than this in the studies conducted by Warditiani, and Andrie (16,13 ± 0,50 %). This previous study could provide higher level of andrographolide because of several step of purifications[38,39,40]. Since the higher level of andrographolide (main active compound), the biological activities of this extract can be increased. Besides, the flavonoids and phenolics contents in EEAP were higher than the previous study[41].

In the study, antihypertensive activity of EEAP was evaluated in phenylephrine-hypertensive rats.

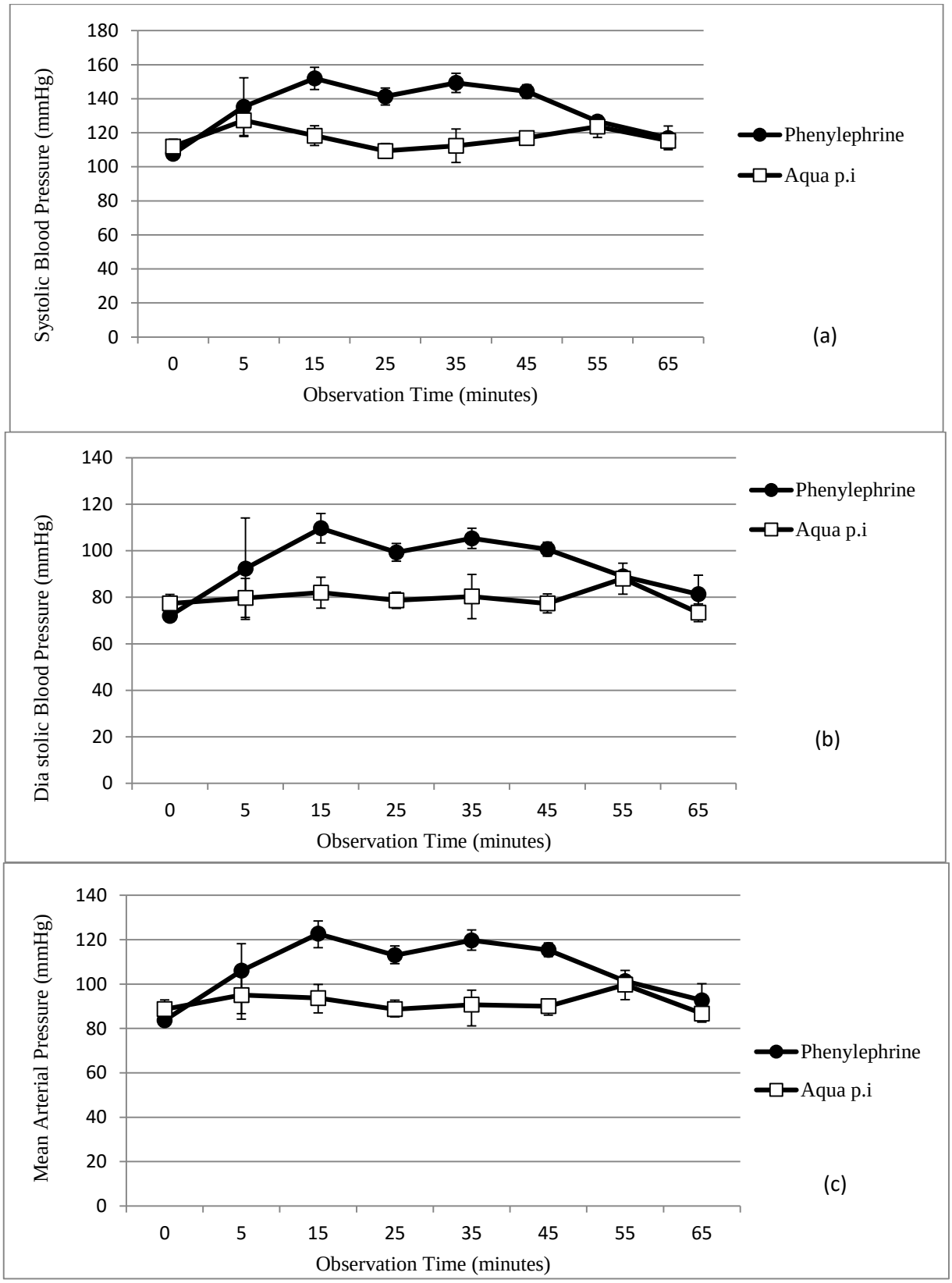

Figure 2. Systolic **(a)**, diastolic **(b)** and mean arterial blood pressure **(c)** profile after either administration of phenylephrine 0.9 mg/kgBW and aquadest. Data were expressed as mean ± SEM (n = 3)

Hypertensive condition was induced by subcutaneous injection of phenylephrine 0.9 mg/kgBW. Phenylephrine is an alpha adrenergic receptor agonist that stimulates vasoconstriction of blood vessels. Activation of the α_1 adrenergic receptor results in vasoconstriction and then increase the blood pressure[41]. In clinical use, phenylephrine is used to increase the blood pressure in shock conditions (anaphylactic shock) and has a duration

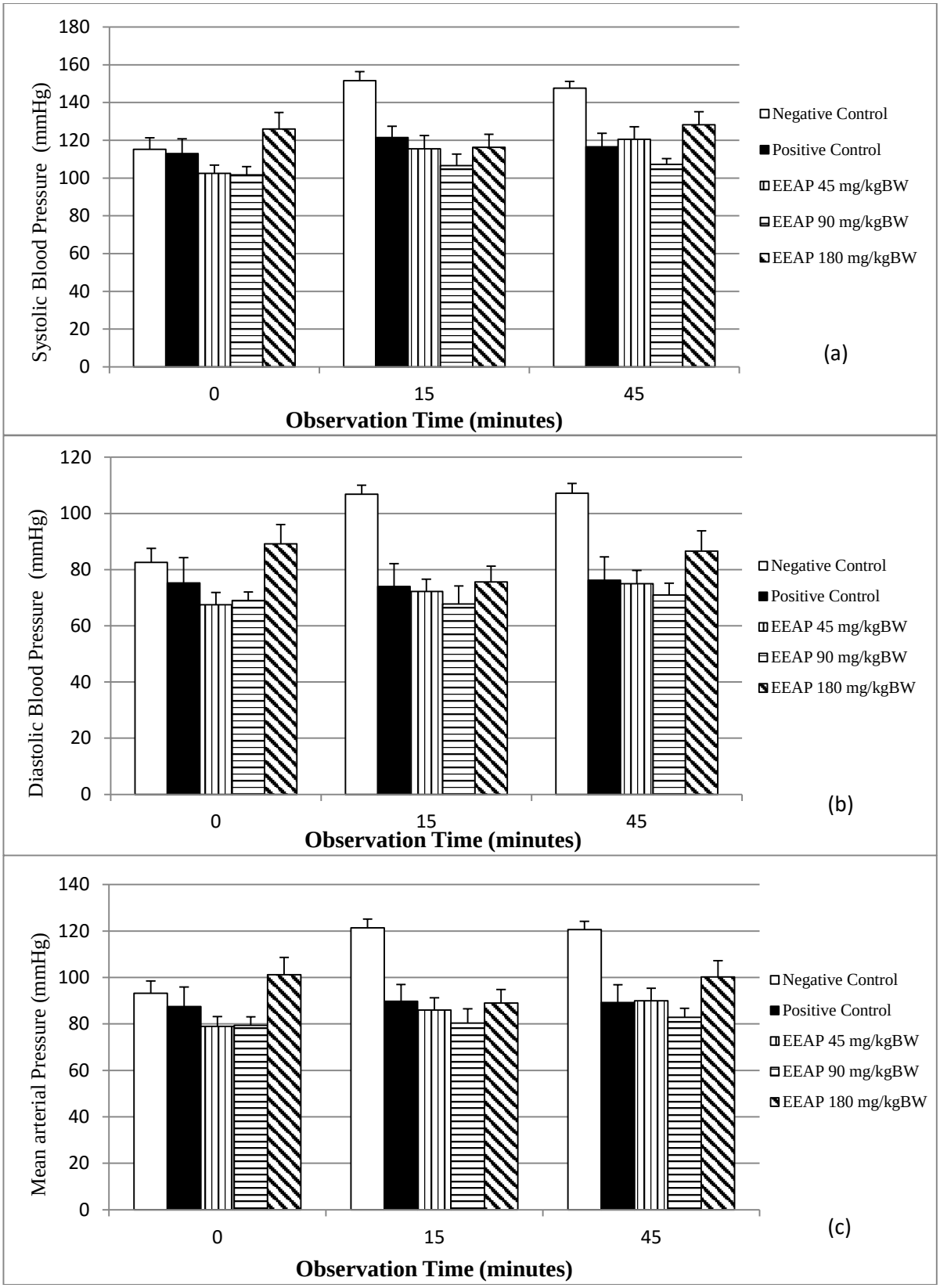

Figure 3. Effect of EEAP administration orally on systolic (a), diastolic (b) and, mean arterial (c) blood pressure after induced with phenylephrine 0.9 mg / kgBW. Negative and positive controls were administration of the vehicle and nifedipine, respectively. Data were expressed as mean ± SEM (n = 4-5)

of 60 minutes. According to The Seventh Report of the Joint National Committee, increase in systolic blood pressure of 20 mmHg and diastolic of 10 mmHg will cause a hypertensive condition.

Table 1: Effects of the vehicle, subcutaneous injection of aqua pi and per oral administration of 1% CMC Na, on blood pressure. Data were expressed as mean ± SEM (n = 3)

Effect of sub-cutaneous injection of aqua pi

Type of blood pressure	Blood pressure (mmHg)		
	0 minute	15 minutes	45 minutes
Systolic	111.00 ±0.58	105.67±1.67*	112.33±2.91*
Diastolic	77.33±4.06	69.33±1.77*	75.00±3.61*
Mean arterial	88.33±2.61	81.00±1.53*	87.00±3.22*

Effect of per oral administration of 1% CMC Na

Type of blood pressure	Blood Pressure (mmHg)		
	0 minute	45 minutes	75 minutes
Systolic	109.33 ±10.60	111.33±11.41*	116.67±6.77*
Diastolic	72.0011.39	70.67±8.22*	73.67±6.97*
Mean arterial	84.00±10.83	84.00±9.55*	87.33±6.65*

* Significant difference (P<0.05) compared to the basal value (min 0)

Table 2: Percentage of decrease in systolic, diastolic, and mean arterial blood pressure after EEAP administration in rats induced by phenylephrine 0.9 mg / kgBW. Data were expressed as mean ± SEM (n = 4-5)

No	Group of test animals	Systolic blood pressure	
		15 minutes	45 minutes
1	Nifedipine	77,34± 6,86%	89,97± 14,33%
2	EEAP 45 mg/kgBW	62,29±7,93 %	44,44 ±23,74%
3	EEAP 90 mg/kgBW	86,26± 12,05%	81, 48± 4,02%
4	EEAP 180 mg/kgBW	115,80± 17,03%*	95,68 ±12,11%
No	Group of test animals	Diastolic blood pressure	
		15 minutes	45 minutes
1	Nifedipine	105,17±10,59 %	95,94±4,98 %
2	EEAP 45 mg/kgBW	80,37±2,60 %	64,43±21,30 %
3	EEAP 90 mg/kgBW	104,96±18,91 %	91,87±9,77 %
4	EEAP 180 mg/kgBW	156,20±22,24 %	110,57±18,49 %
No	Group of test animals	Mean arterial pressure	
		15 minutes	45 minutes
1	Nifedipine	92,02±7,71 %	93,61±7,20 %
2	EEAP 45 mg/kgBW	75,18±5,22 %	59,85±20,91 %
3	EEAP 90 mg/kgBW	96,45±16,11 %	87,59±7,16 %
4	EEAP 180 mg/kgBW	143,26±20,83 %*	103,65±14,75 %

* Significant difference (P<0.05) compared to nifedipine

Evaluation of EEAP have to ensure that its antihypertensive effect only from EEAP and not influenced by its vehicle. The vehicle, 1 % CMC Na did not exhibit any effect on blood pressure. Nifedipine was used as a positive control. The drug is commonly used for treatment of hypertension. Nifedipine acts by blocking Ca^{2+} channel in arterial smooth muscle so cause vasodilatation[42]. In cardiac muscle, Ca^{2+} channel blocker produces negative inotropic resulting in decrease of blood pressure.

In the study, EEAP showed antihypertensive activities including the systolic, diastolic and mean arterial blood pressure. EEAP decreased the blood pressure in phenylephrine-induced rats in dose-dependent manner. Sriramaneni et al reported that chloroform extract of *A. paniculata* at doses of 50 and 100 mg/kgBW given once daily for 4 weeks succeeded to lower the systolic blood pressure in spontaneously hypertensive rats[43]. Zhang et al reported that 14-deoxy-11,12-didehydroandrografolide (DDA), one of diterpene of *A. paniculata* could significantly decrease the mean arterial pressure in anesthetized Sprague Dawley rats[44]. Zhang and Tan evaluated the water extract of *A. paniculata* in anesthetized rats. This extract was able to decrease the mean arterial pressure without decreasing the frequency of the heart rate[45]. Antihypertensive activity of EEAP is contributed by several mechanisms of its active compounds in the extract. Sattayasai et al reported that andrographolide, an active compound of *A. paniculata* herb was able to inhibit the contraction of aorta[46]. The effect of andrographolide is regarding to vasodilatation of blood vessels that occurs due to increase in synthesis of nitric oxide (NO) by endothelial cells[46]. NO is an activator of guanylate cyclase that transform GTP into cGMP. The cGMP acts by facilitatig myosin light chain dephosphorylation so can prevents myosin interaction with actin. This phenomenon is responsible for vasodilation effect[47].

Reportedly, vasodilation effect of andrographolide was related to α-adrenergic receptor blockade[45]. Its alpha-1 adrenergic receptor blockade attenuates the arterial pressure by dilating blood vessels either its resistance or capacitance[47]. According to Burgos et al andrographolide

was calcium channel blocker selective that causes vasodilation by decreasing intracellular Ca^{2+}, which is a major modulator in the activation of myosin light chain kinase[48]. In addition, 14-deoxy-11,12-didehydroandrografolide, an other active compound of *A. paniculata* herbs exhibited antihypertensive effect through β-adrenergic receptor blockade mechanism and angiotensin-converting enzyme inhibitors[44].

This vasodilatation effect was also contributed by the release of nitric oxide from endothelial cells[49]. Antihypertensive mechanism of *A. paniculata* herbs might be contributed by its action in the heart. The 14-deoxy-11,12-didehydroandrografolide and 14-deoxyandrografolide were reported to decrease the cardiac perfusion pressure[50]. Other active compounds besides diterpen in *A. paniculata* were flavonoids and phenolics. Many studies have shown that increase intake of flavonoids and phenolics is correlated with reduced blood pressure and increased cardiac dilatation[51]. The flavonoids are able to induce nitric oxide (NO) on smooth muscle. Induction of endothelial Nitric Oxide can stimulate vasorelaxation effect (52-53). Antihypertensive effect is mediated by nitric oxide guanosine 3 ', 5' cyclic monophosphatepathway (NO-cGMP)[54].

CONCLUSION

Based on the analytical study of EEAP, the extract consisted of andrographolide, total flavonoids and total phenolics by contents of 12.85%, 0.72% and 1.66%, respectively. EEAP has demonstrated antihypertensive activity in phenylephrine-induced hypertensive rats with a non-invasive method. EEAP are potential to develop as an antihypertensive agent in hypertension therapy.

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