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Total phenolic and flavonoid contents, and *in vitro* antihypertension activity of purified extract of Indonesian cashew leaves (*Anacardium occidentale* L.)

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Abstract

Cashew tree (family *Anacardiaceae*) grows widely in many areas of the Southeast Asia countries including Indonesia. Its fruit and nut are used for food, whereas its leaf is one of the traditional antihypertensive medicine in Indonesia. Hypertension affects many people around the world especially in developing countries such as Indonesia. The study aimed to evaluate the *in vitro* antihypertension effect of purified extract of cashew leaves (PECL) using an isolated organ technique, and determine the total phenolic and flavonoid contents. The results showed that PECL at 0.5 and 1.0 mg/mL was obviously able to inhibit the contraction of isolated rat aorta induced by cumulative addition of phenylephrine. The inhibitory effect of PECL were 25.72±8.19% and 39.60±3.50% ($p<0.05$), respectively. PECL (1.0 mg/mL) also changed the pD_2 value of phenylephrine from 6.71±0.37 to 5.93±0.33 ($p<0.05$), and relaxed the isolated-organ mildly by 13.11±0.72%. In addition, PECL contained the total phenolic of 19.78±0.62% and the total flavonoids of 1.97±0.04% which are equivalent to gallic acid and rutin, respectively.

Introduction

Hypertension is a main risk factor of stroke, cardiovascular, and renal failure (August, 2004). This failure was estimated to be 4.5% cause of global disease (WHO and ISH, 2003). Cardiovascular system is affected by arteries that play a role in maintaining the change in peripheral blood flow oscillation. Artery has elastic structures and characteristics (McEniery *et al*., 2007). Disturbance in its elasticity can influence the vascular resistance, one other than cardiac output directly affecting blood pressure (DiPiro *et al*., 2005).

Hypertension can be treated by traditional medicines (Koffi *et al*., 2009). Cashew leaf is one of the traditional antihypertensive medicinal plants in Indonesia which is needed to be developed in the form of purified extract to increase the amount of active compounds. Paris *et al*. (1977) reported that the extract of cashew leaves (ECL) exhibited antihypertensive effect, which was certainly correlated to active chemical compounds contained in the extract. ECL mainly contains phenolic and flavonoid compounds (Paris *et al*., 1977; Syaharuddin *et al*., 1998; Razali *et al*., 2008; Sulaiman *et al*., 2011; Andarwulan *et al*., 2012). Torres-Piedra *et al*. (2011) reported that vasorelaxation effect of traditional medicines were correlated to the content of phenolic and flavonoid compounds. Some epidemiological studies showed inverse correlation between flavonoid consumption and risk of cardiovascular disease (Xu *et al*., 2011). It suggests that antihypertensive effect of ECL might be related to the present of the compounds.

Polyphenolic compounds are widely available in some plants, fruits and vegetables. They are derivatives and/or isomers of flavones, isoflavones, flavonols, catechins and phenolic acids that exhibit cardiovascular protection and improvement of the endothelial function (Han *et al*., 2007). In the previous study, polyphenol-rich cocoa powder exhibited antihypertensive effect in hypertensive rats, eventhough did not influence the arterial blood pressure in the normotensive rats (Cienfuegos-Jovellanos *et al*., 2009). In addition, crude extract of *Terminalia bellerica* fruit that contains flavonoids, sterols and tannins also showed antihypertension

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effects in anaesthetized-rats (Khan and Gilani, 2008).

Development of ECL as antihypertensive medicine was further investigated to obtain a purified extract. The pharmacological effect of purified extract will be more effective because most non-active compounds have been eliminated. Based on the facts, purified extract of cashew leaves (PECL) was investigated for its *in vitro* antihypertension effects and its total phenolics and flavonoids contents. The *in vitro* antihypertension effect was evaluated using an isolated organ technique. Phenylephrine was used to contract the rats-isolated aorta. Total phenolics and flavonoids contents in PECL were determined using Folin Ciocalteu reagent and colorimetric assay, respectively.

Materials and Methods

Materials

The following materials were used :phenylephrine-HCl, gallic acid, rutin from Sigma Chemical Co. (St. Louis, MO, USA). The Krebs solution consists of the following composition (mM) : NaCl 118.0; KCl 4.4; $CaCl_2$ 2.5; $NaHCO_3$ 25.0; $MgSO_4$ 1.1; KH_2PO_4 1.2; glucose 11.0 from Merck & Co., Inc. (New Jersey, USA). The solution must be made freshly, and continuously bubbled with 95% O2 and 5% CO_2.

Animals

Male Wistar rats weighing 200-250 g were obtained from Laboratory of Pharmacology and Toxicology, Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The animal handling protocols of this study were in accordance with the guidelines of the animal care of the Department.

Extraction and purification

The cashew leaves were collected from the area around Plemburan Sleman, Yogyakarta Indonesia and identified by a botanist at Laboratory of Plant Taxonomy, Faculty of Biology Universitas Gadjah Mada Indonesia. The cashew leaves were then dried and powdered. Total 190.5 g of powder was macerated by 70% ethanol for 24 h. Subsequently, the residue was remacerated by same solvent four times, and the extracts were mixed into the previous extract. The collected extract was then evaporated under reduced pressure to give of viscous ethanolic extract, then fractionated with n-hexane yielding soluble and insoluble fractions of n-hexane. The insoluble fraction was concentrated by rotary vacuum evaporator to obtain viscous extract, and then dried using freeze drying to eliminate the existence of the remaining traces of water.

Determination of total phenolic content

Total phenolic content was determined according to the method of Chun *et al*. (2003). Ten milligrams PECL was included in 10 mL volumetric flask, then added with 0.4 mL reagent of Folin-Ciocalteau and incubated for 4-8 min. Furthermore, the solution was added with 4.0 mL of 7% Na_2CO_3, then added with distilled water. After 2 h of incubation, the solution absorbance was measured in 750 nm wavelength versus a blank consisting distilled water and Folin-Ciocalteau reagent. Total phenolic content was expressed in gallic acid equivalent (GAE) of each 100 g PECL of dry weight.

Determination of total flavonoid content

Total flavonoid content was determined according to the procedure of Chang *et al*. (2002) validated by Mujahid (2011) using rutin as reference standard. Both PECL and rutin (100 mg) were dissolved in 10 mL methanol. Subsequently, the routine solution was diluted to provide a series of concentrations (0.050; 0.075; 0.015 and 0.250 mg/mL). The sample solution (0.5 mL) was added with 1.5 mL methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL potassium acetate 1M and 2.8 mL distilled water, and then incubated for 30 minutes. Furthermore, absorbance was measured in 415 nm wavelength, and distilled water with 10% $AlCl_3$ was used as a blank. Total flavonoid content was expressed in gram rutin equivalent (RE) of each 100 gram PECL of dry weight.

Preparation of aortic rat

The isolated organ was rat aorta that represents a vascular system (Nugroho *et al*., 2008), obtained from male wistar rats. The animal was sacrificed by cervical dislocation. The aorta was carefully removed, cleaned and cut (Torres-Piedra *et al*., 2011) into 1 cm length. The strip was mounted in an organ bath containing 20 mL Krebs solution (pH 7.4, 37°C) aerated with 95% O_2 and 5% CO_2. The organ bath was equipped with a level transducer (type 368 HSE, Germany) connected to a bridge amplifier (type 336 HSS, Germany) and a recorder (Kipp & Zonen BBD 41, The Netherlands). The strips was equilibrated for at least 60 min under a resting tension of 1 g. The Krebs solution was replaced with the fresh solution every 15 min.

Evaluation of inhibitory effect on the contraction

After 60-min equilibration, the isolated organ was contracted with a single concentration of phenylephrine. After the contraction of aorta reached

a plateau, the organ was washed by fresh Krebs solution for 60 min. Subsequently, the isolated organ was preincubated with various oncentrations of PECL or DMSO for 5 min, and then contracted with cumulative addition of phenylephrine to provide a concentration-response curve.

Evaluation of vasodilatation effect

In the study, following 60-min equilibration the isolated organ was contracted with a single concentration of phenylephrine. The tissue was washed by the fresh Krebs solution for 60 min with replacement of the Krebs solution every 15 min. The tissue was then contracted with a single concentration of phenylephrine. After the maximun contraction of aorta was reached, cumulative concentrations of PECL (0.1; 0.5 and 1.0 mg/mL) were added into the organ bath to relax the isolated organ.

Statistical and analysis of data

The data were presented as mean ± the standard error of the mean (SEM). In the *in vitro* study, the pD_2 value is a negative logaritmic of concentration of agonist which cause half maximal response in the form of either contraction or relaxation (Waldron *et al*., 1999). That value represents potency of the effect of agonist on the rats isolated-aorta. All data were analyzed statistically using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. P-values less than 0.05 were considered significant.

Results and Discussion

Extraction and purification

In the study, extraction of 190.05 g cashew dry leaves with 70% ethanol yielded 50.79 g viscous ethanolic extract (26.72%) which is accordance with requirements of Farmakope Herbal Indonesia (DepKes RI, 2008). In the purification step, 15.00 g viscous ethanolic extract was fractionated using n-hexane to yield higher contents of active compounds in the fraction. n-hexane was used in the procedure to eliminate chlorophyll, lipid, and other disturbing materials (Depkes RI, 2008; Konan *et al*., 2012). Purification step yielded a viscous, brown coloured fraction with a total weight of 4.27 g (28.47% of weight of the ethanolic extract).

Total phenolic contents

The total phenolic content was determined using Folin-Ciocalteau method. In the study, the absorbances of a series concentrations of gallic acid were plotted to their concentration to yield a linear calibration curve of gallic acid (y = 106.6x + 0.008) with coefficient of correlation (r) value of 0.992 (Figure 1). In the study, total phenolic content of PECL was 19.78±0.62%. It means that each 100 g dry weight of PECL contained total phenolics that was equivalent to gallic acid of 19.78±0.62 g (Table 1). Purification step yielded higher total phenolics content in comparison to ethanolic extract without purification. Related to this case, Sulaiman *et al*. (2011) and Andarwulan *et al*. (2012) reported that ethanolic extract without purification contained the total phenolics content equivalent to gallic acid of 4.36 g/100 g and 0.85 g/100 g dry weight extract, respectively.

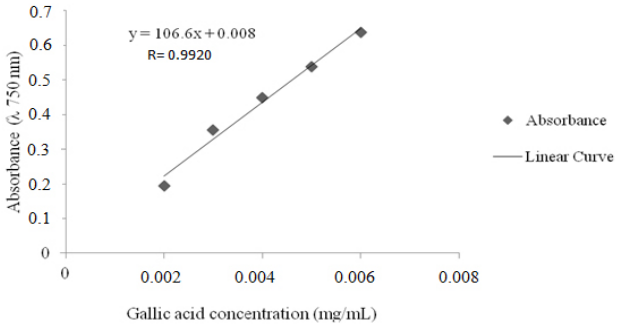

Figure 1. Linear curve of gallic acid concentration (mg/mL) vs. absorbance for determination of total phenolics content

Table 1. Percentage of total phenolic content in the purified extract

Concentration (mg/mL)	Absorbance (n) (λ 750 nm)	% total fenolic content GAE (b/b)
0.004	0.415	19.07
	0.440	20.24
	0.435	20.01
Mean ± SEM		19.78 ± 0.62

GAE = Gallic Acid Equivalent

Total flavonoid content

Flavonoids are the most compounds of plant phenolics. In the study, total flavonoid content was determined using a modified method based on the procedure of Chang *et al*. (2002) validated by Mujahid (2011) using rutin as a reference standard. Principally, the procedure is related to the formation of complex between flavonoid and $AlCl_3$ that produces a yellow coloured solution. The absorbance is measured spectrophotometrically at maximum wavelength of 415 nm (Mujahid, 2011). The total flavonoid content was equivalent to rutin in milligram per gram dry material of the fraction. The absorbances of a series concentrations of rutin were plotted to their concentration to yield a linear calibration curve of rutind (y = 7.96x - 0.074) with coefficient of correlation (r) value of 0.9985 (Figure 2).

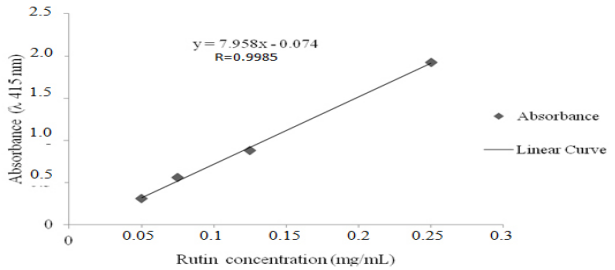

Figure 2. Linear curve of rutin concentration (mg/mL) vs. absorbance for determination of total flavonoids content

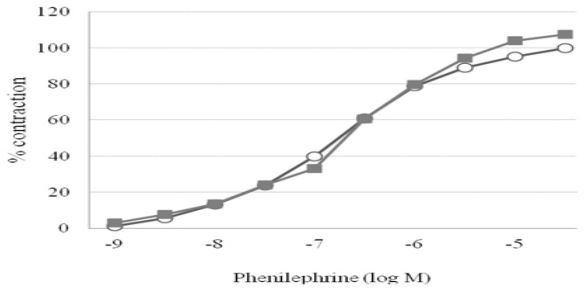

Figure 3. Concentration vs. response curves to Phenylephrine in absence (O) or presence (■) of DMSO in rats isolated-aorta (Data represent mean±SEM, n=4-8)

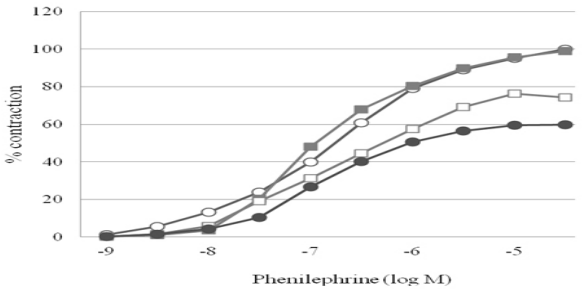

Figure 4. Concentration vs. response curves to Phenylephrine in presence of DMSO (control) (◯), or PECL at 0.1 (■); 0.5 (□) and 1.0 mg/mL (●) in rats isolated-aorta (Data represent mean±SEM, n=4-8)

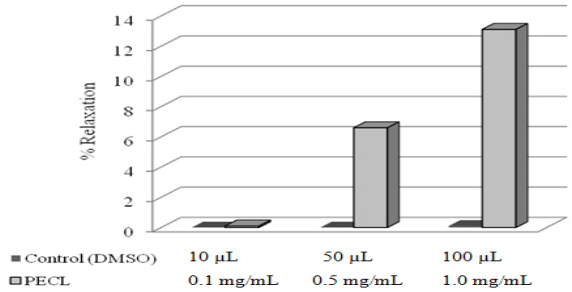

Figure 5. Percentage of relaxation responses after incubated by DMSO (10.0, 50.0, and 100.0 µL) or cumulative addition of PECL (0.1; 0.5 and 1.0 mg/mL)

In the study, total flavonoid content of PECL was 1.97±0.01%. It means that each 100 g dry weight of PECL contained total flavonoid equivalent to rutin of 1.97±0.01 g (Table 2). This total flavonoid content was higher than this of ethanolic extract without purification. In previous study, the leaves were extracted without purification yielding total flavonoid content of 0.93 g/100 g dry weight extract (Sulaiman *et al*., 2011).

Table 2. Percentage of total flavonoid content in the purified extract

Concentration (mg/2 mL)	Absorbance (n) (λ 415 nm)	% total flavonoid content RE (b/b)
21.6	1.613	1.96
	1.623	1.97
	1.621	1.97
Mean±SEM		1.97±0.01

RE = Rutin Equivalent

Inhibitory effects on the contraction of rats isolated-aorta

In the study, rat isolated-aorta was preincubated by either the solvent (DMSO) or PECL, and then contracted gradually by a series concentrations of phenylephrine agonist. The values of pD_2 and maximum contraction responses were compared among these groups. These values represent affinity and intrinsic activity (efficacy) of phenylephrine agonist on the target action (Kenakin, 1997).

Firstly, DMSO which is a solvent used to dilute the fraction (purified extract) was evaluated for its effect on isolated-aorta. In the study, 100 µL DMSO did not influence the maximum contraction of rat isolated-aorta induced by phenylephrine ($P>0.05$). The result indicates that DMSO did not influence the intrinsic activity (efficacy) of phenylephrine agonist. The values of pD_2 of phenylephrine in absence and in presence of 100 µL DMSO were 6.71±0.37 and 6.58±0.27, respectively. Statistically, these values were not significant ($P>0.05$). It indicates that DMSO also did not influence the affinity of phenylephrine.

Secondly, PECL was evaluated for its inhibitory effect on phenylephrine-induced contractions. As shown in Figure 4, PECL decreased the maximum contraction of rat isolated-aorta induced by phenylephrine in concentration-dependent manner. PECL at 0.5 and 1.0 mg/mL suppressed the contraction of isolated-aorta by 25.72% and 39.61%, respectively. The result indicates that PECL decreased the intrinsic activity (efficacy) of phenylephrine agonist. In addition (Figure 4), incubation with PECL also decreased the pD_2 value of phenylephrine. At lower concentration (0.1 mg/mL), PECL did not influence the pD_2 value, however, at 0.5 and 1.0 mg/ml succeeded to shift the pD_2 value of 6.71±0.37 to 6.23±0.24 and 5.93±0.33, respectively. It indicates that PECL was also able to decrease the affinity of phenylephrine. The inhibitory effect of PECL on rat isolated-aorta might be associated with either competitive or non-competitive disruption of the target action of phenylephrine in rat isolated-aorta.

Vasodilatation effects on rats isolated-aorta

In the study, rat isolated-aorta was precontracted by a single consentration of phenylephrine agonist, and after maximum contraction reached, the tissue

was relaxed by a series concentrations of PECL. In the study, the solvent (DMSO) did not influence on rats isolated-aorta. PECL at of 0.1; 0.5 and 1.0 mg/mL relaxed the rat isolated-aorta mildly by 0.1±0.005; 6.64±0.350 and 13.11±0,72, respectively (Fig 5).

Indonesia is a second largest biodiversity country that provides many traditional medicines for various diseases. However, the scientific data are still limited. One of traditional medicine that is interesting to be explored is cashew tree. Cashew (*Anacardium occidentale* L.) is a popular plant in Indonesia due to wide range of applications. Plant parts including leaves, nuts and fruit are used for food, vegetable or traditional medicine. Traditionally, the leaves are used to decrease blood pressure in hypertensive patients. In the study, purified extract of cashew leaves (PECL) that were collected in Indonesia contained total phenolics and flavonoids of 19.78% and 1.97%, respectively. Previously, cashew leaves were also reported containing high amount of phenolic compounds. The alkali- and water-extracts contained gallic acid predominantly, and also protocatechuic, p-hydroxybenzoic, cinnamic, p-coumaric and ferulic acids (Kogel and Zech, 1985). In addition, flavonol glycosides were also identified from the extract of cashew leaves collected in Malaysia. The highest constituent in the extract was kaempferol-3-O-glucoside, followed by kaempferol-3-O-arabinofuranoside and quercetin-3-O-glucoside (Shukri and Alan, 2010). Similarly as in the leaf, the juice of cashew apples collected from Cote d'Ivoire also contained high amounts of total phenolics and total flavonoids. Its bioactive phenolic compounds were such as quercetin, naringenin and phenolic acids (caffeic acid, p-coumaric acid, ferulic acid, gallic acid) (Marc *et al*., 2012). In addition, cashew nuts extracts also contained flavonoid and phenolic compounds. The ethanolic and ethyl acetate extracts only contained phenolics, however acetonic extract contained both compounds (Kannan *et al*., 2009).

Flavonoid and phenolic compounds have widely been reported as antioxidant agents positively correlated in the treatment of cardiovascular diseases due to their antihypertension properties (Akhlaghi and Bandy, 2009; Xu *et al*., 2011). Some traditional medicine containing high amounts of flavonoid and phenolic compounds exhibit antihypertension activity. Reportedly, polyphenol-rich cocoa powder obviously produced antihypertensive effect in hypertensive rats, but did not influence the arterial blood pressure in the normotensive rats (Cienfuegos-Jovellanos *et al*., 2009). In addition, crude extract of *Terminalia bellerica* fruit containing flavonoids, sterols and tannins markedly decreased the arterial blood pressure of anaesthetized rats. The extract also inhibited the force and rate of atrial contractions in isolated guinea-pig atria, and also relaxed the phenylephrine and K^+-induced contractions in the Ca^{2+}-free medium (Khan and Gilani, 2008). Several mechanisms that may contribute to their antihypertension properties are such as the inhibition of phosphodiesterase, reduction of intracelluler Ca^{2+}, induction of nitric oxide (NO) in smooth muscles (Akhlaghi and Bandy, 2009; Torres-Piedra, 2011). Among them, NO induction from vascular endothelium resulting from antioxidative activity of flavonoid and phenolic compounds have an important contribution in their antihypertension properties.

Nowadays, oxidative stress is still an attractive target for cardiovascular prevention and therapy. The fact is related to the abnormal production of reactive oxygen species (ROS) producing decrease in vascular bioavailability of NO that contributes in the pathogenesis of the endothelial dysfunction in the development of hypertension disease (Munzel *et al*., 2010; Cohen and Tong, 2010). In the production of ROS, many vascular enzymes are involved such as NADPH oxidases, xanthine oxidase, and uncoupled endothelial nitric oxide synthase. Compounds that are potent inhibitors for oxidative stress are considered to be used in hypertension therapy due its vascular function preservation activity (Weseler and Bast, 2010). Polyphenols from fruits, vegetables and beverages can protect the cardiovascular system due to their antioxidative and free radical scavenging activities. The compounds are able to generate the NO from vascular endothelium, and then prevent the endothelial dysfunction that contribute mainly in the pathogenesis of hypertension (Curin and Andriantsitohaina, 2005). Reportedly, acetonic extract of cashew leaves exhibited a poten antioxidative activity determined by DPPH method (Vijayakumar and Kalaichelvan, 2011). In addition, the ethanolic extract also showed antioxidant activities in the DPPH and nitric oxide radical scavenging assay. The high amount of phenolic compounds in the extract of cashew leaves positively contribute to the antioxidative activity (Jaiswal *et al*., 2010).

In the study, purified extract of cashew leaves (PECL) exhibited inhibitory effect on phenylephrine-induced contractions. The potent antioxidative effect of cashew leaves due its flavanoids and phenolics may underlie its antihypertension effect. Further investigation is needed to explain the molecular mechanism of PECL on its antihypertension activities. In this case, phenolic and flavonoid compounds might play main roles in the antihypertension effects needed to be isolated and further investigated for their target

actions.

Conclusion

Purified extract of cashew leaves which contains higher contents of flavonoid and phenolic compounds exhibited antihypertension effects in rats isolated-aorta. The purified extract is potential to develope as an antihypertension agent.

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