



Metabolomic study on the effect of Indonesian propolis in hypertensive rats

[Estudio metabolómico del efecto del propóleo indonesio en ratas hipertensas]

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Abstract

Context: Hypertension is a health problem that is a major factor triggering cardiovascular diseases; however, effective treatment has not been found.

Aims: To evaluate the effectiveness of Indonesian propolis in hypertension treatment through metabolomic study.

Methods: A total of 36 Sprague Dawley rats were divided into 6 groups, including standard diet group, high-NaCl diet group, high-NaCl diet + captopril group (25 mg/kg), high-NaCl diet + propolis from Riau Archipelago group, high-NaCl diet + propolis from Lampung group, high-NaCl diet + propolis from South Sulawesi group. An 8% NaCl diet was used to induce hypertension for 3 weeks, while 200 mg/kg propolis was administered for 1 week following the induction. The bioactive compounds of ethanol extract of propolis were identified using UHPLC. Moreover, the metabolomic profile was performed using LC-MS/MS. Data was processed using principal component analysis and was used for metabolic pathway analysis.

Results: All propolis samples significantly ameliorated the blood pressure of hypertensive rats. The metabolites that were mainly found in rat serum were amino acids, glycerophospholipids, and acylcarnitine. Rats administered with propolis from South Sulawesi had the closest metabolite profile to the standard group, where L-acetylcarnitine could be the potential marker of hypertension. Several metabolic pathways, including the degradation of tryptophan, β -fatty acid oxidation, degradation of threonine, and 2-oxobutanoic acid, were the most impaired pathways in hypertension.

Conclusions: Indonesian propolis, especially from South Sulawesi, could be used for controlling blood pressure.

Keywords: biomarkers; blood pressure; hypertension; metabolomics; propolis.

Resumen

Contexto: La hipertensión es un problema de salud que constituye un importante factor desencadenante de enfermedades cardiovasculares; sin embargo, no se ha encontrado un tratamiento eficaz.

Objetivos: Evaluar la eficacia del propóleo indonesio en el tratamiento de la hipertensión mediante un estudio metabolómico.

Métodos: Un total de 36 ratas Sprague Dawley fueron divididas en 6 grupos, incluyendo el grupo de dieta estándar, el grupo de dieta alta en NaCl, el grupo de dieta alta en NaCl + captopril (25 mg/kg), el grupo de dieta alta en NaCl + propóleos del archipiélago de Riau, el grupo de dieta alta en NaCl + propóleos de Lampung y el grupo de dieta alta en NaCl + propóleos de Sulawesi del Sur. Se utilizó una dieta con un 8% de NaCl para inducir la hipertensión durante 3 semanas, mientras que se administraron 200 mg/kg de propóleos durante 1 semana tras la inducción. Los compuestos bioactivos del extracto etanólico del propóleo se identificaron mediante UHPLC. Además, se realizó el perfil metabolómico mediante LC-MS/MS. Los datos se procesaron mediante análisis de componentes principales y se utilizaron para el análisis de rutas metabólicas.

Resultados: Todas las muestras de propóleos mejoraron significativamente la presión arterial de las ratas hipertensas. Los metabolitos que se encontraron principalmente en el suero de las ratas fueron aminoácidos, glicerofosfolípidos y acilcarnitina. Las ratas administradas con propóleos de Sulawesi meridional presentaron el perfil de metabolitos más parecido al del grupo estándar, en el que la L-acetilcarnitina podría ser el marcador potencial de la hipertensión. Varias vías metabólicas, como la degradación del triptófano, la oxidación de los ácidos β -grasos, la degradación de la treonina y el ácido 2-oxobutanoico, fueron las vías más alteradas en la hipertensión.

Conclusiones: El propóleo indonesio, especialmente el procedente de Sulawesi meridional, podría utilizarse para controlar la presión arterial.

Palabras Clave: biomarcadores; hipertensión; metabolómica; presión arterial; propóleos.

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INTRODUCTION

Hypertension is one of the most dangerous public health problems as it is the major risk factor for cardiovascular diseases, such as heart attack, heart failure, stroke, and kidney disease (Hoffmann et al., 2020). It is estimated that nearly 1.3 billion adults worldwide lived with hypertension in 2019 (Zhou et al., 2021). Meanwhile, more than one-third of adults in Indonesia also have hypertension, according to a recent national report (Berretta et al., 2020). Several metabolic changes are found in hypertension, including dyslipidemia, hyperinsulinemia, as well as vitamin and amino acid metabolic disturbances. Balancing these changes may become the target for the effective treatment (Gao et al., 2019).

Propolis, a resinous substance collected by bees, has been evaluated as an anti-hypertensive agent through different mechanisms depending on its bioactive compound (Silva et al., 2015). Propolis from several countries, including Brazil, Australia, Tunisia, and Indonesia, have been reported to demonstrate anti-hypertensive activity in animal studies (Gargouri et al., 2019; Maruyama et al., 2009; Mulyati et al., 2021). However, the explanation of the underlying mechanism of propolis in controlling blood pressure is limited. Dihydrokaempferide, kaempferide, butelolol, isosakuranetin, and caffeoylquinic acid were reported to be responsible for blood pressure control (Maruyama et al., 2009). However, the knowledge of the metabolic pathway of propolis in reducing blood pressure is still inconclusive (Wang et al., 2020). Our previous study proposes that the effect of propolis on hypertension through different mechanisms depending on the type of propolis, either through a diuretic effect or improving blood lipid profile (Mulyati et al., 2021). Another study showed the effect of propolis on controlling blood pressure via vasodilation by increasing the bioavailability of nitric oxide (NO) (Vaziri, 2004).

Metabolomics study has been extensively used to diagnose a disease early and explain the molecular mechanisms of drugs, disease treatments, and prevention (Johnson and Gonzalez, 2012). Over the past 20 years, metabolomics has been widely applied to disease management, e.g., cardiovascular disease, diabetes, inherited metabolic disorders, alcohol-induced liver disease, cancer, and pharmacokinetic studies (Johnson and Gonzalez, 2012). In hypertension, the metabolomic study showed several alterations in metabolic pathways, including pyruvate, lactic acid, valine, tryptophan, alanine, aspartate, glutamate, and arginine. These alterations are closely related to insulin resistance, inflammatory state, and impaired NO

production (Deng et al., 2021). Exploring the metabolic pathway of propolis on blood pressure control enables us to better understand the effect of propolis in hypertension prevention. Therefore, this study aimed to evaluate the potential use of Indonesian propolis in hypertension treatment through a metabolite profiling study using hypertensive rats.

MATERIAL AND METHODS

Chemicals and reagents

Extraction of propolis used 70% ethanol, which was diluted from 96% ethanol (Merck, Darms tadt, Germany). HPLC-grade formic acid, acetonitrile, and methanol (Merck, Darms tadt, Germany) were used for HPLC and LC/MS-MS analysis, respectively. For hypertension induction, the present study used NaCl (Merck, Darms tadt, Germany). Moreover, captopril was purchased from a local pharmacy.

Propolis extraction

Propolis samples were obtained from local beekeeper in Riau Archipelago (1°3'39.3696" N, 104°14'12.0732" E, Lampung (5°29'35.6820" S, 105°4'44.8464" E.), and South Sulawesi Province (GPS coordinates: -2°59'33.00" S, 20°11'48.98" E), Indonesia. Propolis from Riau Archipelago, Lampung, and South Sulawesi were manufactured by *Trigona itama*, *Geniotrigona thoracica*, *Tetragonula biroii*, respectively. All samples were collected in September 2020 and kept under -20°C until used. Extraction of samples was performed using 70% ethanol, with the ratio between the sample and the solvent was 1:10. The extraction was assisted by ultrasonication method. Propolis was cut into small pieces (0.5–1 cm) and was dissolved in the solvent. Furthermore, ultrasonication (Ultrasonic Cleaner GA-008, Granbo, China) was applied for 3 h at room temperature with a power of 35 Watts and frequency of 30 kHz. The mixture was kept in the freezer for 24 h and then filtered using Whatman filter paper No. 41 to obtain a clear filtrate. The filtrate was then evaporated using a rotary evaporator (Buchi, Flawil, Switzerland) at 40°C to produce a dry extract (Fikri et al., 2018).

Identification of bioactive compounds of propolis

The analysis of bioactive compounds was conducted using UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS (Thermo Scientific™, Waltham, MA, USA). The column used was C18 accucore phenyl hexyl, 100 mm × 2.1 mm × 2.6 μm, where the column was programmed at 40°C with a flow rate of 0.20 mL/min. The analysis used a linear gradient solvent system consisting of solvent A:B (0.1% formic acid in

H₂O: 0.1% formic acid in acetonitrile), where t = 0 min, 95% solvent A; t = 3 min, 75% solvent A; t = 7 min, 40% solvent A; t = 10 min, 20% solvent A; t = 13 min, 100% solvent B; t = 15 min, 5% solvent B. Eluted compounds were detected using electrospray ionization (ESI) from m/z 100 to 1500 in positive ionization mode. The capillary and cone voltages were 0.8 kV and 30 V, respectively. The injection temperature was 120°C with the desolvation and cone gas flow rate at 1000 L/h and 50 L/h, respectively.

Animals

A total of 36 Sprague Dawley rats aged 7-8 weeks and weighed 250-300 g were used. Acclimatization was done for 2 weeks, and the animals were caged under standard laboratory conditions (temperature between 22-25°C; 12:12 light: dark cycle). Sodium chloride (NaCl) was used to induce hypertension following the procedure of (Aoki et al., 2013). The rats were divided into 6 groups (n = 6 per group): SD group received a standard diet; NaD group received a high NaCl (8%) diet (SD); PD group received a high NaCl (8%) diet + captopril (25 mg/ kg); NaDP1 received high NaCl (8%) diet + propolis from Riau Archipelago (200 mg/kg); NaDP2 received high NaCl (8%) diet + propolis from Lampung (200 mg/kg); and NaDP3 received high NaCl (8%) diet + propolis from South Sulawesi (200 mg/kg). A high NaCl (8%) diet was administered for 3 weeks to produce hypertensive rats (systolic blood pressure >140 mmHg). The previous study concluded that a dose of 200 mg/kg effectively reduced hypertension (Selamoglu Talas, 2014). Moreover, for ethical reasons, we only used one dose as the main objective of this study was to compare the effectiveness of propolis samples from several provinces of Indonesia and to explore the metabolomic changes after the administration of propolis. The measurement of blood pressure followed the method of Malkoff using a non-invasive blood pressure system (CODA, Kent Scientific, USA) (Malkoff, 2005).

The rats were administered with either propolis or captopril for 1 week, respectively. After three weeks of the hypertension induction and one week of intervention, the animals were euthanized, and blood serum was collected for metabolomic analysis. The blood serum was put into a microtube and mixed with cold methanol. The mixture was homogenized using a vortex for 1 min then it was centrifuged at 6000 rpm and 4°C for 5 min. The separated solution was obtained and stored in a refrigerator at ± 4°C. All experimental procedures were conducted in accordance with and approved by the Animal Care and Use Committee, IPB University (No. 176-2020 IPB).

Metabolomic analysis using LC-MS/MS and principal component analysis (PCA)

Identification of metabolites in blood serum referred to the method of Wang et al. with some modifications (Wang et al., 2020). A total of 5 µL serum was injected to LC-MS/MS equipped by C18 accucore™-Phenyl Hexyl LC Column (2.1 mm × 100 mm × 2.6 µm) (Thermo Scientific, Germany). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) with the following gradient concentrations: 98-2% A (0-17 min) and 2-98% B (0-17 min). The ESI parameters were set with a spray voltage of 3.8 kV, collision energy of 18, 35, 53 eV, sheath gas flow rates of 3, aux gas flow rates of 12, and capillary temperature of 320°C. The mass scan range was between m/z 80 to 1200. The power resolution was set to 70000 FWHM. Furthermore, the metabolite profiles were identified using the Compounds Discoverer 3.2 (Thermo Fisher, Waltham, USA). The identification was performed by selecting the spectra, aligning the retention time, and detecting the compound according to the databases. Moreover, the Unscramble X software was used for PCA to investigate the metabolic profile differences between healthy and hypertensive rats. PC score plotting was used to visualize the PCA data.

Statistical and metabolic pathway analysis

Data were expressed in mean ± standard deviation and the number of samples. The blood pressure differences between pre and post-intervention were determined by paired t-test. Furthermore, the change differences between the groups were analyzed using ANOVA with the Tukey post-hoc test. SPSS version 21 was used to perform the test with significance at p<0.05. In addition, pathway analysis used MetaboAnalyst website (<https://www.metaboanalyst.ca/>) with database sources including Kyoto Encyclopedia of Genes and Genome (KEGG) Compound Database (<http://www.genome.jp/kegg/compound/>) and Small Molecule Pathway Database (SMPDB) (<https://www.smpdb.ca>). The websites were used to evaluate the construction, interaction, and pathway analysis of potential biomarkers.

RESULTS

Bioactive compound identification

The results of the bioactive compounds identification using LC-MS/MS showed that propolis extract from Riau Archipelago, Lampung, and South Sulawesi contained 22, 22, 26 compounds, respectively. The identified compounds were mainly polyphenolic groups (flavonoids, flavones, flavonols, and chalcones), several terpenoids, and fatty acids and their de-

rivatives (Table 1). Fig. 1 shows the chromatogram of the LC-MS/MS results from the three propolis extracts.

Effect of propolis on blood pressure of rats

Fig. 2 shows the blood pressure changes during the treatments. The results showed that a high NaCl diet (8%) successfully caused hypertension in rats after three weeks of administration. Moreover, all propolis samples (200 mg/ kg) significantly reduced systolic and diastolic blood pressure after 1 week of intervention ($p < 0.05$). The changes were similar to those of the captopril (25 mg/ kg) group.

Metabolomic profile of rat serum and PCA results

The metabolomic profile showed a total of 27 metabolites found in rat serum. These metabolites were mainly amino acid group, glycerophospholipids, lysophospholipids, acylcarnitine, keto acids, and carboxylic acids, where amino acids, glycerophospholipids and acylcarnitine were predominantly found. The metabolite compounds found using the in-house database were integrated into HMDB (Human Metabolome Database), KEGG, PubChem, and the ChemSpider databases.

Biplot analysis showed that the grouping between the samples was quite clear (Fig. 3). The study

demonstrated that the metabolite profile of rats administered with propolis extract from South Sulawesi seemed to be closer to rats with a standard diet (healthy rat) and captopril group. This may indicate that the administration of propolis extract from South Sulawesi could provide the most effective treatment for hypertension by considering its similarity in metabolite profile. The loading plot analysis showed that the position of L-acetylcarnitine (1) was quite different from that of the main group, indicating this compound as the potential marker for hypertension treatment. Besides L-acetylcarnitine (1), the other compounds, including L-threonine (4) and serotonin (7), could also participate in explaining the effect of propolis extract from South Sulawesi on hypertension.

Metabolic pathway analysis

Metabolic pathway analysis using enrichment analysis indicated that the metabolic pathway of tryptophan, β -oxidation of very long-chain fatty acids, degradation of threonine and 2-oxobutanoic acid, and oxidation of branched-chain fatty acids showed the highest alteration in hypertension (Fig. 4). The metabolites of each pathway were L-tryptophan, serotonin, L-acetylcarnitine, L-threonine, and L-palmitoyl-carnitine, respectively.

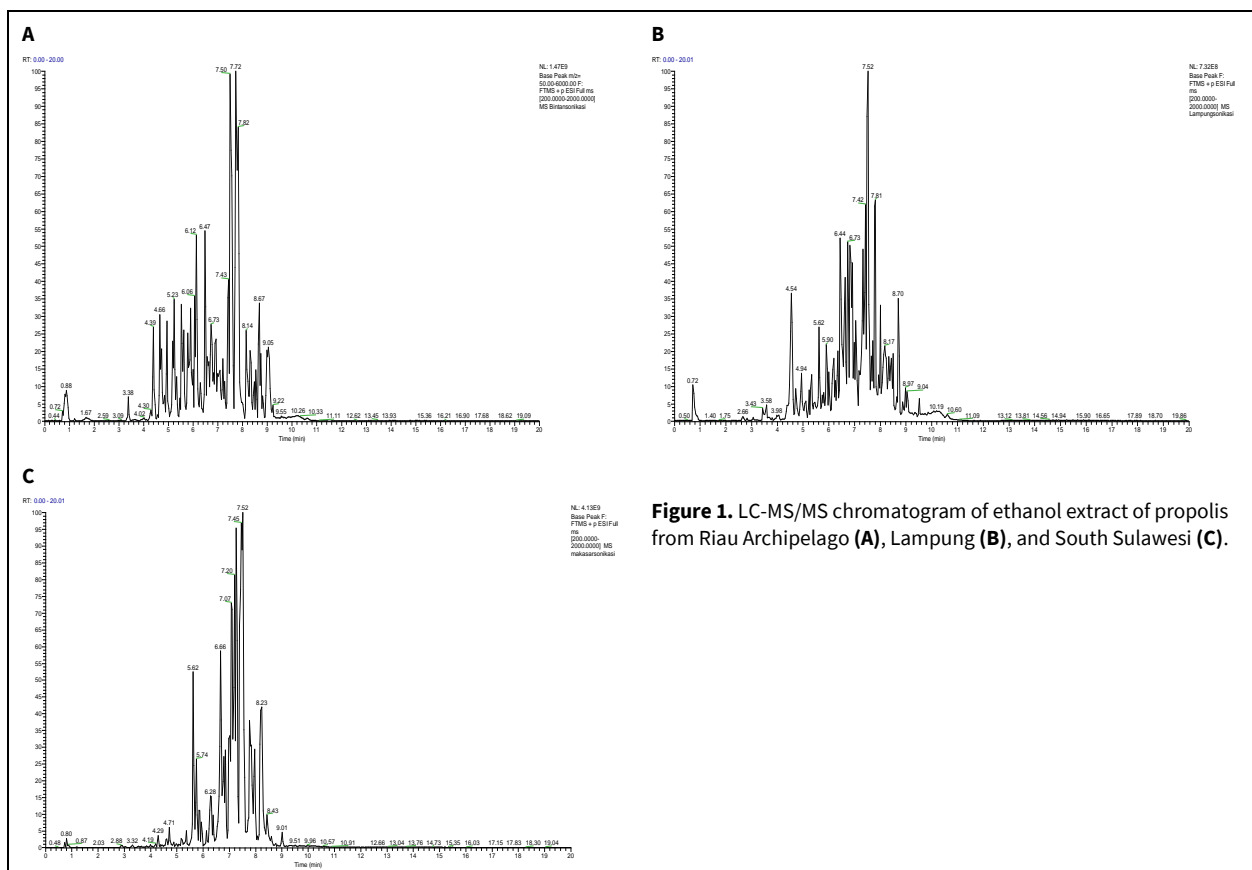
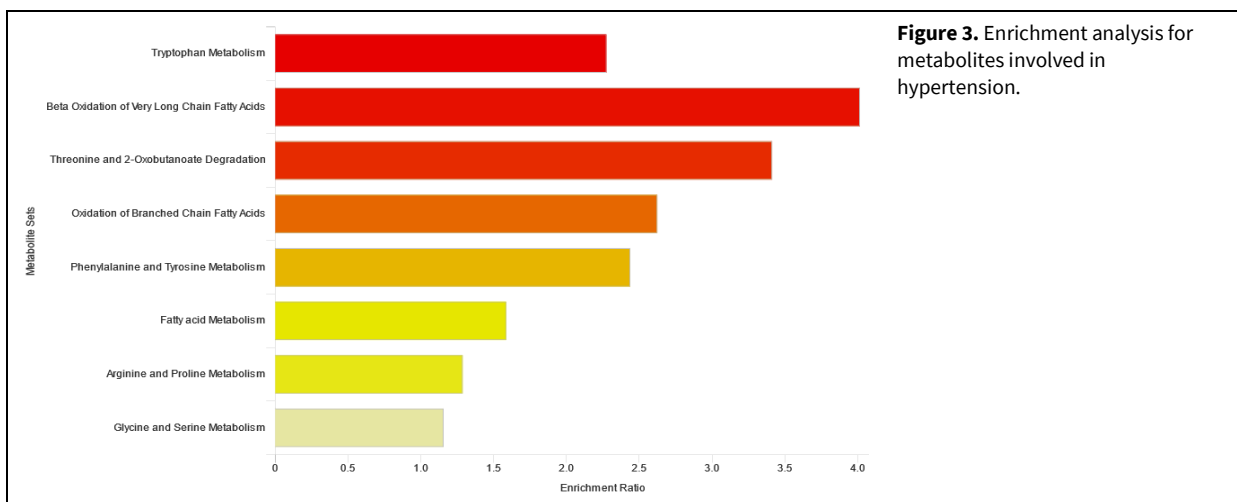
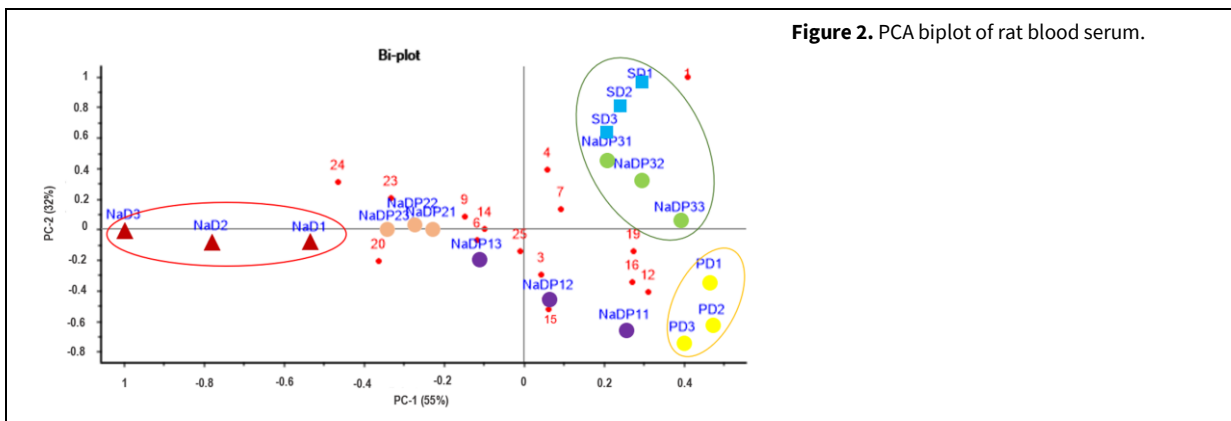
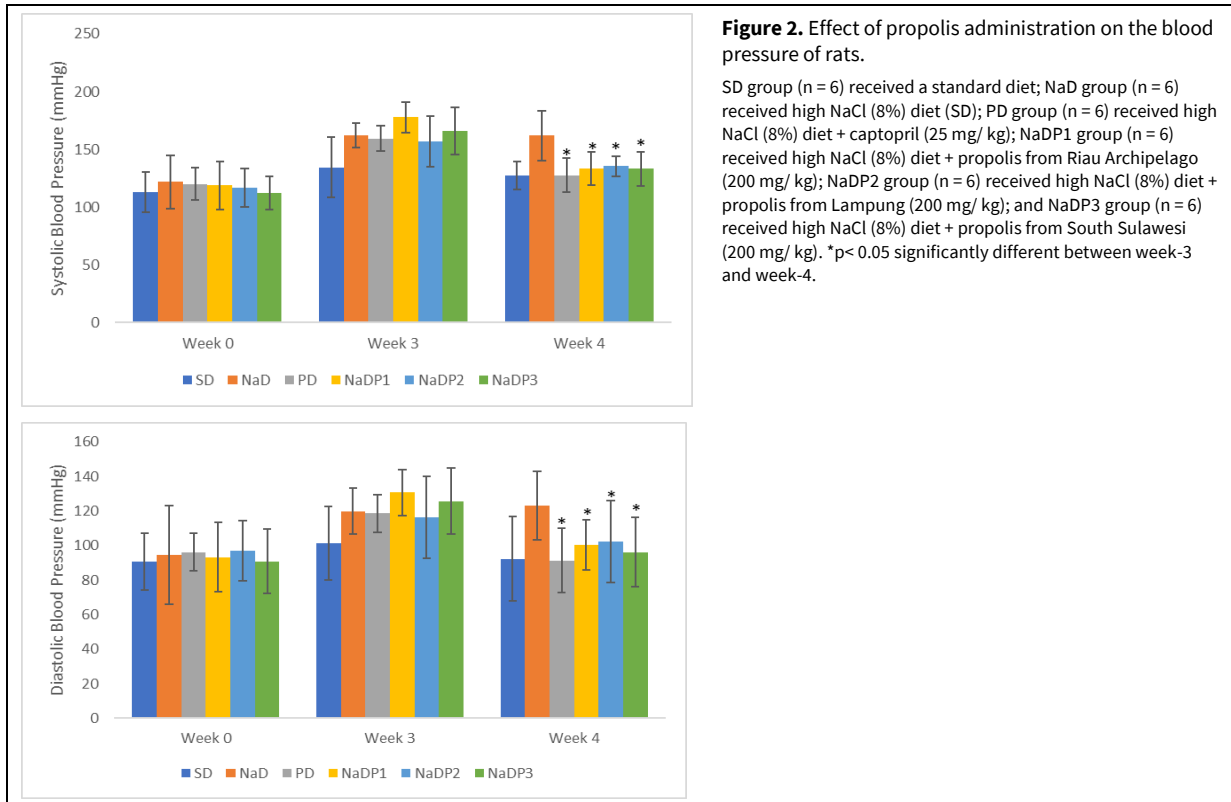


Figure 1. LC-MS/MS chromatogram of ethanol extract of propolis from Riau Archipelago (A), Lampung (B), and South Sulawesi (C).

Table 1. The bioactive compounds of the ethanol extract of propolis from Riau Archipelago, Lampung, and South Sulawesi.

No.	Compound	Group	Propolis		
			Riau Archipelago	Lampung	South Sulawesi
1	Bonannione A	Flavanone	+	+	+
2	Kaempferide	Flavonol	+	+	+
3	Propolin A	Prenylflavonoid	+	+	+
4	Macarangin	Flavone	+	+	+
5	Acacetin	Flavone	-	+	+
6	7-O-Prenylpinocembrin	Flavonoid	+	-	+
7	Apigenin	Flavone	+	+	+
8	Ferulic acid methyl ester	Ferulic acid derivative	-	+	-
9	Naringenin	Flavanone	+	+	+
10	Farnesyl acetate	Sesqui diterpenoid	-	-	+
11	Lupeol	Triterpenoid	+	+	+
12	Isorhamnetin	Flavonoid	+	+	+
13	Hesperetin	Flavanone	+	-	+
14	Pinocembrin	Flavanone	+	+	
15	(S)-4-methoxydalbergione	Neoflavonoid	-	+	+
16	Neoflavonoid 8	Flavonoid	+	-	+
17	Luteolin	Flavone	+	+	+
18	(3S)-Violanone	Flavonoid	+	-	-
19	Dihydrocinnamic acid methyl ester	Esther derivate	-	+	-
20	Solophenol A	Prenylflavonoid	+	+	+
21	(-) Caryophyllene oxide	Sesquiterpene	-	+	+
22	5,7,3',4'-Tetrahydroxy-5'-C-geranylflavanone	Flavanone	+	+	+
23	Neoflavonoid 1	Neoflavonoid	-	-	+
24	Quercetin	Flavonol	+	+	+
25	Stearic acid	Fatty acid	-	+	-
26	b-Amyrin acetate	Triterpenoid steroid	-	-	+
27	Odoratin	Sesquiterpene	+	-	+
28	2',6'-Dihydroxy-4',4'-dimethoxydihydrochalcone	Chalcone	-	-	+
29	Medicarpin	Isoflavonoid	-	+	-
30	Ellagic acid	Polyphenol	-	-	+
31	Chrysin	Hydroxyflavone	-	-	+
32	Oleic acid	Fatty acid	+	+	+
33	Neoflavonoid 10	Flavonoid	+	-	-
34	4,4'-Dihydroxy-2'-methoxychalcone	Chalcone	+	-	-
35	(2S)-7-Hydroxyflavanone	Flavanone	-	+	-
36	(3S)-Vestitone	Flavonoid	+	-	-



DISCUSSION

Our results showed that the propolis samples mainly contained polyphenolic compounds. Polyphenolics, including flavonoids, flavones, flavonols, and chalcones, are the typical bioactive compounds found in propolis (Gargouri et al., 2019). The previous study also found that polyphenolics were the main constituent of Indonesian propolis from several provinces (Fikri et al., 2018; 2019). These compounds may be responsible for several biological activities, including antioxidant, antibacterial, antiviral, antifungal, immunomodulatory, anti-inflammatory, anticancer, antidiabetic, and even anti-hypertension properties. All samples were harvested in September, a transitional season between rainy and dry seasons in Indonesia. This month could be an adequate time to harvest propolis with optimum chemical composition, as the previous studies showed the composition of phenolics, flavonoids, and diterpenes reached during or after summer (Isla et al., 2012).

The present study revealed that all Indonesian propolis significantly reduced the blood pressure of hypertensive rats after one week of administration. The reductions were quite similar to those of the captopril group, indicating that the propolis samples remarkably controlled the blood pressure. These findings were in line with the previous study reporting that propolis notably decreased the blood pressure of hypertensive animals (Maruyama et al., 2009). Several isolated compounds, including dihydrokaempferide, kaempferide, butelelol, isosakuranetin, and caffeoylquinic acid, have been proposed to be responsible for the anti-hypertensive properties of propolis (Maruyama et al., 2009). However, the mechanism from a metabolic perspective was still unclear.

Our metabolomic study found that the metabolite profile of rats administered with propolis extract from South Sulawesi was similar to the standard group. This may suggest that propolis from South Sulawesi could give an effective treatment in balancing the metabolite profile of hypertensive patients. Our previous report also showed propolis from South Sulawesi demonstrated high biological activities, including antituberculosis (Sulaeman et al., 2018), anti-emesis (Fikri et al., 2018), and immunomodulator (Fikri et al., 2022). Compared to other Indonesian propolis, propolis from South Sulawesi was reported to have the highest antioxidant activity and flavonoid contents (Fikri et al., 2019). Antioxidant activity of propolis has been thought to underlie the mechanism of anti-hypertensive activity of propolis by increasing the bioavailability of NO (Sinha and Kumar Dabla, 2015). Recently, a study from Lombok, Indonesia, found that a similar composition of propolis from

South Sulawesi, originating from *Calophyllum* resin, showed significant antibacterial activity (Mizuno et al., 2022). In addition, our previous studies successfully observed that the flora surrounding propolis from the Riau Archipelago were coconut and acacia, while those in Lampung were *Mangifera*, silk tree, jackfruit, and Hevea tree (Nusa et al., 2020).

PCA results showed that L-acetylcarnitine may become the potential biomarker for hypertension treatments. L-acetylcarnitine is a metabolite involved in the process of β -oxidation of very long-chain fatty acids, which is the main fuel source of ATP synthesis. L-acetylcarnitine plays a role in importing fatty acyl-CoA to mitochondria for oxidation. Previous studies have observed a close relationship between fatty acid metabolism and hypertension. An abnormal concentration of L-acetylcarnitine has been reported in a recently published hypertension study (Mey et al., 2020). Moreover, some studies showed dyslipidemia was in concurrence with hypertension incidence (Tang et al., 2022). High LDL and low HDL have been observed in patients with hypertension. In hypertension, the level of L-acetylcarnitine was lower, probably due to high energy consumption in the blood circulation. A study shows carnitine treatment can significantly increase nitric oxide synthase (NOS) (Alanazi et al., 2020). Moreover, the previous study showed that propolis improved the blood lipid profile of rats, which supported the anti-hypertensive effect of propolis through lipid metabolism (Mulyati et al., 2020).

The metabolic pathway analysis found that several other pathways, including the metabolism of tryptophan, serotonin, and threonine, were also impaired in hypertension. Our findings align with the recent report showing that pyruvate, tryptophan, and some other amino acids are the consistent biomarkers of hypertension (Deng et al., 2021). L-tryptophan and serotonin are involved in tryptophan metabolism and contribute to pulmonary artery smooth muscle proliferation, vasoconstriction, and microthrombosis (Chen et al., 2020). Serotonin is produced from 5-hydroxytryptophan with the activity of tryptophan decarboxylase. Furthermore, serotonin acetylase converts serotonin into N-acetylserotonin, which is a precursor of melatonin (Wang et al., 2020). Melatonin is an anti-hypertensive hormone due to its anti-inflammatory activity and reactive oxygen species (ROS) scavenging activity (Ott et al., 2019). Also, L-threonine and 2-oxobutanoic acid are the metabolite products of threonine breakdown, which can also be converted into pyruvate. In hypertension, threonine degradation might be impaired due to the participation of pyruvate in an inflammatory state and oxida-

tive stress (Deng et al., 2021). Considering that the metabolic alterations in hypertension are mainly due to oxidative stress, we ultimately conclude that the antioxidant activity of propolis plays a significant role in ameliorating blood pressure.

CONCLUSION

The bioactive compounds contained in Indonesian propolis from Riau Archipelago, Lampung, and Sulawesi were mainly the groups of polyphenolics (flavonoids, flavones, flavonols, and chalcones), terpenoids, and fatty acids and their derivatives. All propolis samples significantly decreased blood pressure in hypertensive rats. A total of 27 metabolites were identified in our animal models, including amino acids, glycerophospholipids, lysophospholipids, acylcarnitines, keto acids, and carboxylic acids. PCA showed that the metabolite profile of rat serum administered with propolis from South Sulawesi had the closest metabolite profile to the standard group, indicating the most effective treatment. L-acetylcarnitine might become the potential marker of hypertension. The results of the metabolic pathway analysis revealed that the degradation of tryptophan, β -fatty acid oxidation, degradation of threonine, and 2-oxobutanoic acid were the most affected metabolic pathways in hypertension. Hence, we believe that the antioxidant activity of propolis plays a significant role in relieving hypertension.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Mulyati AH	Yanti HD	Warnasih S	Sulaeman A	Rafi M
Concepts or ideas	x			x	
Design	x	x	x	x	x
Definition of intellectual content		x	x	x	
Literature search	x			x	x
Experimental studies	x			x	x
Data acquisition	x	x	x	x	x
Data analysis	x	x	x	x	x
Statistical analysis	x	x	x	x	x
Manuscript preparation	x	x	x	x	x
Manuscript editing	x	x	x	x	x
Manuscript review	x	x	x	x	x

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