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RESEARCH ARTICLE

Assessing the antioxidant properties of *Pilea trinivera* (Roxb.) Wight) by in vitro and in vivo studies

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Abstract

Background: Pohpohan leaves (*Pilea trinivera* (Roxb.) Wight), commonly consumed as fresh vegetables, contain flavonoids useful as antioxidants. Literature studies showed the content of quercetin, kaempferol, myricetin, luteolin, and apigenin. **Objective:** The research aimed to determine the antioxidant activity of 70% ethanolic extract of Pohpohan leaves in Wistar rats induced by heat stress with the parameters malondialdehyde (MDA) and superoxide dismutase (SOD). **Method:** The pilot study found the extract's total flavonoid, phenolic content, and IC50, respectively, 1.48%, 1.64%, and 79.65ppm. The sample consisted of 30 male Wistar rats that met the criteria and were divided into six groups. MDA levels were determined using the TBARs method, while SOD activity was measured using the spectrophotometric method. Data analysis was done using the Mann-Whitney test. **Result:** MDA levels (nmol/ml) for normal control, negative control, positive control, doses I, II and III group, respectively, were 2.581; 12.978;0.830; 8.597; 6.041; 2.291 whereas the SOD activity (U/ml) of each group was 161.55; 101.54; 267.70; 184.62; 207.69; 240. There were no significant differences in MDA and SOD levels between the positive control group and dose III. **Conclusion:** The results showed the antioxidant activity of the ethanol extract of *Pilea trinervia* (Roxb.) Wight).

Introduction

The emerging cases of mortality caused by Non-Communicable Diseases (NCDs) continue to be on the rise. In 2019, 74% of mortality was caused by NCDs (World Health Organisation, 2020). NCDs can be caused by several factors, such as oxidative stress, often associated with the manifestation of a pathological state (Crisóstomo *et al.,* 2022). Oxidative stress happens when free radicals outnumber antioxidants in the body (Sinaga, 2016). The amounts of free radicals in the body accumulate from cell metabolisms and external factors (Lobo *et al.*, 2010).

Free radicals are molecules with a free electron in the outermost orbit, which are highly reactive and unstable and, therefore, could cause chain reactions that lead to cellular damage (Wahdaningsih *et al.,* 2011). Free radicals can be classified as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Wahjuni *et al.,* 2015). ROS causes oxidative damage to lipid biomolecules by lipid peroxidation, where cell membranes react with ROS (Wahjuni *et al.,* 2015). These processes result in the formation of toxic substances such as malondialdehyde (MDA) (Zaetun *et al.*, 2019). MDA is commonly used as a marker in in-vivo lipid peroxidation because its elevation is constant with oxidative stress and is a specific product of lipid peroxidation (Mulianto, 2020).

Heat stress is caused by the accumulation of heat in the body that surpasses the body's thermoregulation ability (Tarigan *et al.,* 2018). Heat stress causes the elevation of free radical precursor superoxide anion (Slimen *et al.,* 2014). This could alter DNA, protein denaturation, and inflammation response (Alfadda & Sallam, 2012). As a mechanism of protection, the body

regularly produces endogen antioxidants such as the superoxide dismutase (SOD) enzyme (Simanjuntak & Zulham, 2020). SOD works as a catalyst turning superoxide anion into hydrogen peroxide, which will be further processed by catalase and glutathione (GSH) peroxidase into water molecules and oxygen (Fukai & Ushio-Fukai, 2011). Previous studies of pohpohan leaves found the leaves to contain ascorbic acid and phenolic compounds and, therefore, can be used as antioxidants (Violeta & Kumala, 2017).

Methods

Design

Pilot study

This study used a 70% ethanol extract of P. *trinervia* acquired from BALITTRO (Indonesian Medicinal and Aromatic Crops Research Institute). The extract underwent phytochemical screening, determination of total flavonoid and phenolic compounds using UV-vis spectrophotometry (Shimadzu UV-1800), and testing for antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) against vitamin C.

In vivo study

This study used 30 male wistar rats between the ages of two to three months and weighing 150-200g. The animals were divided into six groups, namely: normal control (no heat stress), negative control (heat stress 70 minutes before blood collection), positive control (given Glisodin on the 8^{th}-15^{th} day and 70 minutes of 43°C heat stress two hours after last Glisodin induction), dose (given 70% ethanol extract of *P. trinervia,* I: 100mg/kg BW, II: 200mg/kg BW, and III: 400mg/kg BW on the 8^{th} -15^{th} day and 70 minutes of 43°C heat stress two hours after last extract induction). The UPN Veteran Jakarta Health Research Ethics Committee validated animal usage with agreement number 166/IV/2021/KEPK.

Blood collection was done on the 15^{th} day from the rat's sinus orbitalis. Blood samples were centrifuged at 3000rpm for ten minutes to obtain the red blood cells and collected into a new tube to determine MDA concentration. Following the previous process, add 0.5ml NaCl 0.9% into the tube and repeat the centrifugation; after removing the top layer, 1ml of distilled water was added to the tube to be centrifuged. This process will result in a clear yellowish liquid on the top layer; the plasma is then collected and stored for the determination of SOD activity.

Assessment

Pilot study

Phytochemical screening used the Farnsworth method to catalogue the secondary metabolite content of 70% ethanol extract of *P. trinervia*. The total flavonoid contained in the extract using UV-vis spectrophotometry was λ=425nm, whereas the total phenolic contained in the extract using UV-vis spectrophotometer was λ=769nm. To understand the antioxidant activity of the extract, this study used DPPH as a free radical and vitamin C as a positive control. This study used a UV-vis spectrophotometer (λ=515 nm) 45 minutes after incubation.

In-vivo study

MDA concentration was determined using the Thiobarbituric acid reactive substance (TBARs) method. A standard curve was plotted using 1,1,3,3-tetraethoxypropane in a series of concentrations (0, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 nmol/ml), then into each tube 1ml of trichloroacetic acid 20% and 2ml of thiobarbituric acid 0.67% were added. The tubes were incubated in a water bath (100°C) for ten minutes, and the tubes that changed colour into purplish red were analysed using a UV-vis spectrophotometer (λ=532 nm; 37°C). To analyse the concentration of MDA in a red blood cell sample, 1ml of 20% trichloroacetic acid and 2 ml of 0.67% thiobarbituric acid were added into 200µL of red blood cells and incubated for ten minutes in a water bath (100°C).

SOD activity was done following the instruction sheet from Randox Laboratories Ltd. Diamond Road, Crumlin, Co. Antrim, UK. SOD in plasma samples would inhibit the formation of formazan red dye from xanthine oxidase (XOD) and 2-4-iodophenyl-3-4-nitrophenyl-5-phenyltetrazoliumchloride (I.N.T.). One unit of SOD is responsible for 50% of inhibition formazan red dye formation. The sample was analysed using a UV-vis Spectrophotometer (λ=505 nm; 37°C), and absorbance was recorded on the 30th and 180^{th} second. MDA level and SOD activity were then plotted into tables according to their group. Statistical data analysis was done using Kruskal-Wallis followed by the Mann-Whitney method with a confidence interval 0.05.

Results

Pilot study

Phytochemical screening of ethanol extract of *P. trinervia* using Farnsworth's method showed that the extract has alkaloids, flavonoids, saponins, gallotannins and catechutannins, steroids/terpenoids, cummarine

and essential oils content. Total flavonoid and phenolic content were determined using spectroscopy (λ=425 nm; 769 nm), showing the average total flavonoid and phenolic content in the extract are 1.48±0.02% and 1.64±0.01%, respectively. The antioxidant activity of *P. trinervia* extract was compared to vitamin C to determine the IC_{50} of DPPH. The spectroscopy (λ=515.0 nm; t=45 m) shows the average IC_{50} of *P. trinervia* extract and vitamin C is 79.65 and 4.79 ppm.

In vivo study

The Malondialdehyde (MDA) concentration measured in blood plasma showed the highest concentration in the negative group, 12.9779±2.345 nmol/ml. The concentration decreases at Dose I, II, III i.e. 8.5972±2.724, 6.0414±1.826, 2.2905±1.296 nmol/L, respectively. The positive group has the lowest MDA level, 0.8303±0.971 nmol/L, while the normal group was 2.5812±0.789 nmol/L. Percentages of reduction in MDA for Dose I, Dose II and Dose III from the negative group, respectively, are 33.8%, 53.5%, and 82.3%. The mean of each group was calculated and plotted into a graph shown in Figure 1.

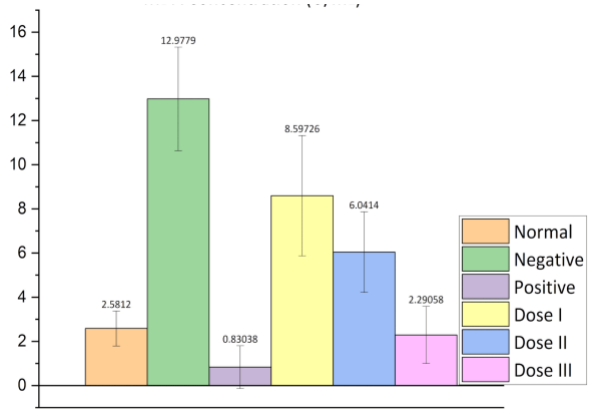

Figure 1: MDA concentration (U/ml)

The SOD activity obtained from haemolysed blood showed that a higher dose of P. *trinervia* extract (Dose III) has higher activity of SOD, followed by Dose II, and Dose I, namely 239.992±12.659, 207.69±0.000, 184.62±0.000 U/ml, respectively. The positive group has the highest SOD activity, i.e. 267.702±12.635. Meanwhile, the negative group has the lowest, 101.538±12.635 U/ml. SOD activity for the normal group is 161.55±0.000 U/ml. Percentages of increase in SOD for Dose I, Dose II and Dose III from the negative group, respectively, are 81.8%, 104.5% and 136.4%. The mean of each group was calculated and plotted in Figure 2.

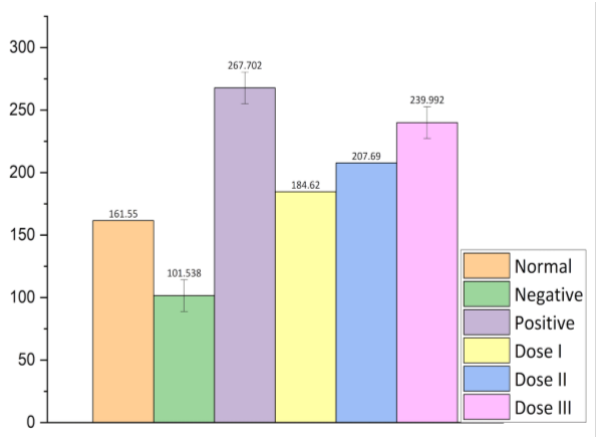

Figure 2: SOD Activity

Statistical analysis conducted on MDA concentration and SOD activity showed significant differences in each group. Fisher's LSD, used to understand the significance between groups, showed that the Dose III group, which was given 400mg/kg BW of extract, had no significant difference to the positive group (p = 0.213) and the normal group (p = 0.801). The full observation of significance is shown in Table I.

Table I: Level of MDA and SOD activity

Group	Level of MDA (U/ml)	SOD Activity (U/ml)
Normal	2.5812±0.789^{c}	161.55±0$^{\#,x}$
Negative	12.9779±2.345^{a,e}	101.538±12.635^{*,x}
Positive	0.8303±0.971^{c}	267.702±12.635$^{*,\#}$
Dose I	8.5972±2.724^{a,d,e}	184.62±0$^{*,\#,x}$
Dose II	6.0414±1.826^{b,c,e}	207.69±0$^{*,\#,x}$
Dose III	2.2905±1.296^{c}	239.992±12.659$^{*,\#,x}$

Data presented as mean±SD. Level of significance for MDA: a = 0.000 to the normal group; b = 0.006 to the normal group; c = 0.000 to the negative group; d = 0.001 to the negative group; e = 0.000 to the positive group. Level of significance for SOD: * = 0.000 to the normal group; # = 0.000 to the negative group; x = 0.000 to the positive group.

Normal group: no heat stress; negative group: heat stress induction; positive group: Glisodin on 8-15th day, induction of heat stress; Dose I: 100mg/kgBW; Dose II: 200mg/kgBW; Dose III: 400mg/kgBW of extract on 8-15th day, induction of heat stress.

Discussion

This study used male wistar rats aged two to three months. Male rats are chosen to acquire a more stable result because it will not be altered by hormonal cycle and pregnancy like in female rats. Faster metabolism in Wistar rats is important for studying the effects of test substances.

Heat exposure over a prolonged period of time accumulates in the body due to the inability to regulate heat, causing heat stress (Tarigan *et al.,* 2018). The accumulated heat results in the formation of superoxide anion from nicotinamide adenine dinucleotide (NADH), which increases the ubiquinone mutants (Slimen *et al.*, 2014). Superoxide anion reacts with lipid biomolecules, causing lipid peroxidation and contributing to oxidative stress (Wahjuni *et al.,* 2015).

The TBARs method is used as an indicator to observe oxidative damage and the presence of free radicals in measuring MDA levels. Two molecules of TBA and one molecule of MDA in the optimal environment (acidic; T = 97-100°C) will form a TBA-MDA complex with chromophore groups that have a purple colour. Absorbance is then measured using a UV-Vis spectrophotometer (λ= 532nm) to determine the concentration of MDA in blood samples (Aspamufita & Yuliani, 2013). To measure the SOD level, this enzyme needed to be released from its cell or organelle (Slimen *et al.,* 2014). The measurement uses spectrophotometry to observe the inhibition of reaction catalysed by superoxide radicals.

After the induction of Glisodin as the positive control and P. *trinervia* extract as the test substance, the results showed lower MDA and higher SOD levels than the negative control, which shows the antioxidant ability of both substances. Glisodin works by enhancing the SOD activity therefore lowering the MDA level. This shows that Glisodin can inhibit lipid peroxidation. Secondary metabolites contained in *P. trinervia* extract responsible for antioxidant activity are alkaloids, flavonoids, saponins and tannins.

Alkaloids work as free radical scavengers, where the alkaloids' indole group efficiently inhibits free radicals' chain reaction (Anggraito *et al.,* 2018). Flavonoids can transfer one electron to free radicals and form a complex with metals, enabling flavonoids to inhibit lipid peroxidation and tissue damage caused by free radicals (Yuhernita & Juniarti, 2011). Flavonoids also inhibit the formation of ROS by inhibiting the activity of xanthine oxidase and nicotinamide adenine dinucleotide phosphate (NADPH) (Lubis, 2018). Flavonoids also indirectly enhance the gene expression of SOD through nuclear factor erythroid two related factor 2 (Nrf2) (Jawi & Sumardika, 2012). Saponins enhance the formation of SOD and catalase (Anggraito *et al.,* 2018). Whereas the OH group in tannins inhibits superoxide (O_2^-), peroxyl (ROO^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), nitrite oxide (NO^-) and peroxynitrite ($ONOO^-$) in the body (Anggraito *et al.*, 2018).

Conclusion

P. trinervia extract has secondary metabolite content, which has strong antioxidant activity. In vivo study using MDA and SOD parameters, *P. trinervia* extracts with a concentration of 400mg/kgBW showed no significant difference with positive control.

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