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Original Article
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Pandanus amaryllifoius Roxb. Leaves Ethanol Extract Ameliorates Lipid and Proinflammatory Cytokines Profiles in a Rat Model of Dyslipidemia

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Objectives: Dyslipidemia has currently become a major health challenge that still opens for safer and more effective modes of treatment. The plant *Pandanus amaryllifolius* Roxb. (pandan) has been indicated to contain active ingredients that interfere with the pathological pathway of dyslipidemia. The aim of the study was to test the effects of pandan leaves ethanol extract on lipid and proinflammatory profiles in a rat dyslipidemic model.
Methods: Dyslipidemia was induced by administration of high-fat feed for 8 weeks. Treatments (vehicle, the reference drug simvastatin at 1.8 mg/kg, and extract at 200, 300 or 600 mg/kg) were given for 4 weeks following the completion of induction.
Results: Significant post-treatment decreases in total cholesterol, low density lipoprotein (LDL), and triglyceride levels in groups receiving all doses of extract and simvastatin were observed. Similar results were also found in regards to proinflammatory cytokines levels. Pandan extracts significantly lowered the concentrations of IL-6, TNF-α, and NFκB p65. Characterization of metabolite contents of the extract confirmed the presence of the previously suggested active alkaloids pandamarilactonine-A and B.
Conclusion: Taken together, results of the present study implied the ameliorating effects of pandan leaves ethanol extract in dyslipidemic condition which is potential for opening an avenue in combating this essential component of metabolic disorder.

Keywords: *Pandanus amaryllifoius* Roxb., pandan, leaves, ethanol extract, dyslipidemia, rats

INTRODUCTION

Metabolic disorders are non-communicable diseases with an increasing prevalence over the years; they entail considerably high healthcare costs in terms of prevention and treatment [1]. Their risk factors include unhealthy eating patterns, obesity, lack of physical activity, and alcohol and tobacco consumption [2, 3].

One of the metabolic disorders is dyslipidemia, which is characterized by increased levels of total cholesterol, low-density lipoprotein-cholesterol, and triglycerides and decreased levels of high-density lipoprotein-cholesterol or their combinations [4]. It is a risk factor for life-threatening myocardial infarction and stroke, which are mediated by atherosclerosis formation [5]. The development of atherosclerosis has been linked to proinflammatory cytokines, whose inhibition can interfere with its pathophysiologic pathway [6].

With the increasing public awareness of the harmful effects of synthetic drugs, medicinal plants have been increasingly used in the treatment of various diseases [7]. For example, the aromatic pandan (*Pandanus amaryllifolius* Roxb.) plant has been used as fragrance in cuisine and as a food colorant. Several studies have shown that its pharmacological activities include antihyperglycemic [8], antimicrobial [9], antiviral [10] and hepatoprotective [11] activities. However, few studies [12] have explored its antidyslipidemic activity. Using the *in silico* para-

digm, we recently found the potency of its alkaloid content by interacting with HMG-CoA reductase, PPAR alpha, NPC1L1, which are essential in lipid biosynthesis and transport [13].

In the present study, we further investigated the antidyslipidemic activity of pandan leaf ethanol extract in a rat model. We observed the changes in blood lipid parameters and measured proinflammatory cytokine levels. We also assessed the inhibition of HMG-CoA reductase *in vitro* and determined the possible active ingredient.

MATERIALS AND METHODS

1. Extract preparation

Pandan leaves were obtained from Bandung, West Java, Indonesia, and identified at the School of Life Sciences and Technology Institut Teknologi Bandung (certificate no. 232/IT1.C11.2/TA.00/2022). Fresh pandan leaves were washed under running water, cut into small pieces of 2-3 cm, and dried in an oven at 40℃. The dried leaves were ground with a blender, and extract was prepared using 96% ethanol with a ratio of 1 part of ground leaves to 10 parts of ethanol. The system was let to sit with periodic stirring for 3 days. The extract was filtered and concentrated using a rotary evaporator at 40℃. The concentrated pandan leaf ethanol extract was stored in a light-protected container at 4℃ prior to its use in subsequent tests.

2. Animal model of dyslipidemia and treatment

An antidyslipidemia activity test was performed using male Wistar rats (School of Life Sciences and Technology, Institut Teknologi Bandung) aged 2-3 months and weighing 150-180 g. They were acclimatized at 25 ± 2℃ and with a 12 h:12 h light–dark cycle for 14 days in the animal laboratory of the School of Pharmacy, ITB. They were given food and drink *ad libitum*. The care and use of the test animals were approved by the Animal Ethical Committee of ITB (certificate no. 06/KEPHP-ITB/2-2022). The rats were divided into six groups: normal group, negative control (vehicle), positive control (1.8 mg/kg simvastatin), and treatment groups (treated with 200, 300, and 600 mg/kg extract). Dyslipidemia was induced by administering high-fat feed containing 200 mg/kg cholesterol, 0.1% cholic acid (p.o), and 12.5 mg/kg propylthiouracil (p.o) for 8 weeks by the time the dyslipidemic model was developed. Afterward, all treatments were given for 4 weeks.

3. Phytochemical screening

The extract was qualitatively screened to detect the presence of flavonoids, saponins, tannins, alkaloids, steroids, and triterpenes.

4. Characterization of the metabolite contents of the extract

This procedure was carried out as previously described [14]. In brief, 1.5 mg of the extract was added with methanol and sonicated for 10 min until it was completely dissolved. The sample was filtered with a 0.22 μm PTFE syringe filter (Waters, Milford, Massachusetts, USA) until a concentration of 1 mg/mL was obtained. Liquid chromatography–mass spectrometry (LC–MS) analysis was performed using a Waters Xevo-G2 XS QTof with a Waters BEH C18 1.8 μm (50 mm) column in an MSn positive sensitivity polarity mode. The solvents were acetonitrile (B) and 0.1% formic acid dissolved in water (A). The starting gradient was 5% B held for 1 min; its concentration was gradually increased to 100% B for 10 min and maintained in this stage for 3 min. The system was returned to the initial solvent composition for 3 min until column equilibrium was reached at a flow rate of 0.3 mL/min. Each measurement was compared with a blank, and the volume injected was 1 μL. Measurement results were analyzed using UNIFI version 1.5 software, and peaks were compared tentatively with the Water built-in the library. The following MS conditions were set: column temperature, 40℃; mass range, 100-1,200 Da; cone voltage, 30 V; capillary, 2 kV; source temperature, 120℃; desolvation temperature, 500℃; cone glass flow, 50 L/h; desolvation gas flow, 1,000 L/h; and collision energy, 10-40 eV.

5. Determination of total phenol and flavonoid

Total phenol was determined using Folin–Ciocalteu reagent under alkaline conditions, measured colorimetrically at 730 nm, and expressed as milligrams of gallic acid equivalent (GAE) per gram of extract. The total flavonoid content was also measured colorimetrically at 425 nm upon reaction with aluminum chloride under a slightly acidic condition. Results were expressed in milligrams of quercetin equivalent (QE) per gram of extract.

6. Inhibitory activity of HMGCR *in vitro*

This test was based on the activity of the extract to inhibit

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HMGCR enzyme activity *in vitro* by using a Sigma-Aldrich HMGCR assay kit. A concentration series of the extract (1.25, 2.5, 3.75, 5, 6.25, and 7.5 ppm) was prepared using high-purified water as a solvent. Each extract solution was reacted with NADPH, HMG-CoA substrate, and phosphate buffer; then, the HMGCR enzyme was added. Afterward, absorption measurements were conducted at 340 nm wavelength and 37℃ for 10 min with an interval of 20 s between each measurement. Pravastatin and phosphate buffer were used as a reference and a negative control, respectively. The percentage of HMGCR inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\frac{-\Delta A_{340\,nm}}{\Delta T}\text{(Enzyme)} - \frac{\Delta A_{340\,nm}}{\Delta T}\text{(Enzyme + inhibitor)}}{\frac{-\Delta A_{340\,nm}}{\Delta T}\text{(Enzyme)}}.$$

7. Analyses of serum lipid levels

Upon treatment completion, the rats were subjected to 14 h of fasting with water *ad libitum* and then euthanized. After they were sacrificed, blood samples were collected via intracardiac puncture. Serum was separated from the blood samples and collected through centrifugation at 2,500 rpm for 10 min. Then, it was incubated at −20℃ for further analysis. Total cholesterol, low density lipoprotein (LDL), and triglycerides in the serum were determined using a test kit (Glory Diagnostics®).

8. Measurement of proinflammatory cytokines

The expression levels of IL-6, TNF-α, and NFκβ p65 in serum were determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Elabscience®). Analyte concentrations were analyzed using a BMG Labtech SPECTROstar Nano Microplate Reader spectrophotometer.

9. Data analyses

Data were analyzed using Statview and expressed as mean ± SEM. Pre- and post-treatment lipid profiles were compared using paired t-test, while the effects of treatment on proinflammatory cytokine levels were assessed using one-way ANOVA with *post hoc* PLSD. The criterion for statistical significance was $p < 0.05$.

RESULTS

1. Phytochemical screening

The phytochemical screening of pandan leaf extract revealed positive results for flavonoids, phenolics, saponins, tannins, alkaloids, and steroids/triterpenes (Table 1). This qualitative characterization has been commonly used as a general method to predict the metabolite contents of a biologically active natural product. Data further serve as a guide for the isolation of the active ingredient responsible for a pharmacological effect.

2. Characterization of the metabolite content of the extract

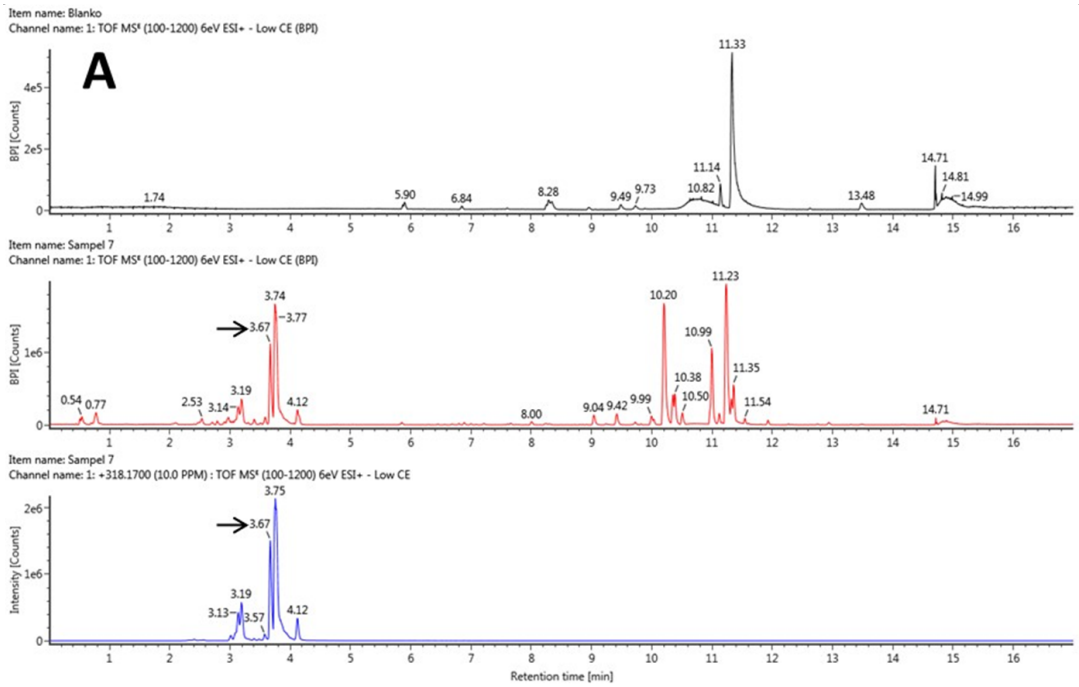
The results of of LC-HRMS of the crude extract are shown in Fig. 1. The three highest peaks were obtained at retention times of 3.67, 3.74, and 10.20 min with the molecular formula of *m/z* at 318.1716 $(M + H)^+$ (calculated for $C_{18}H_{24}NO_4$, 318.1705, Δm = 3 ppm), *m/z* at 318.1705 $(M + H)^+$ (calculated for $C_{18}H_{24}NO_4$, 318.1705), and 10.20. The Dictionary of Natural Product database (DNP) (https://dnp.chemnetbase.com, accessed on 23.01.2024) was searched using *Pandanus* as the limiting keyword for biological source, and 65 compounds were found. Further search using the chemical formula $C_{18}H_{23}NO_4$ as the second limiting keyword for the peaks at retention times of 3.67 and 3.74 min revealed 12 possible compounds corresponding to those peaks; specifically, all of them were pandamarilactonine A or B and its epimers. Therefore, we tentatively concluded that pandamarilactonine A or B and [15] its epimers were present in the crude extract. The mass spectrum of pandamarilactonine A or B and its epimers is depicted in Fig. 1.

3. Total phenol and flavonoid

The total phenol and flavonoid contents were 31.76 mg/g

Table 1. Phytochemical screening results

Metabolites	Results
Flavonoid	+
Phenolic	+
Saponin	+
Tanin	+
Alkaloid	+
Steroid; triterpene	+



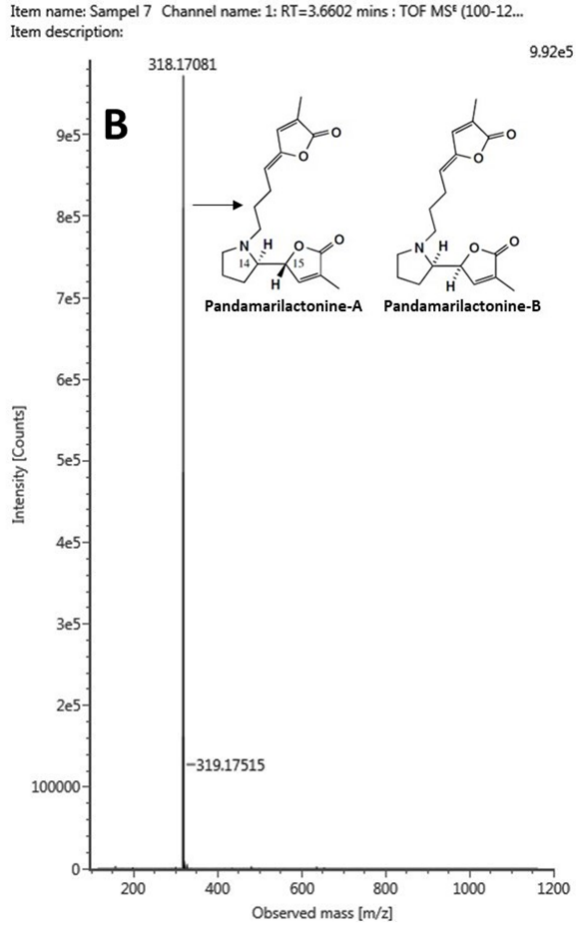


Figure 1. LCMS chromatogram of pandan leaves extract. (A) Base peak chromatogram of blank and pandan leaves extract, with the suggested antidyslipidemic alkaloids pandamarilactonine-A or B (arrows); (B) Mass chromatogram of pandamarilactonine A or B.

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extract QE and 80.91 mg/g extract GAE, respectively.

4. HMGCoA reductase inhibitory activity

The measurement of the HMGCoA reductase inhibitory activity showed that standard pravastatin and extract had IC_{50} of 0.072 and 3.159 μg/mL, respectively.

5. Lipid serum levels

High-fat feed induction for 8 weeks significantly increased the levels of the measured lipid parameters in serum. Further comparisons between the levels of lipid parameters at the end of induction and upon treatment completion showed that 200, 300, and 600 mg/kg pandan leaf extracts significantly reduced the total cholesterol (15.92%, 14.46%, and 34.73%, respectively; Fig. 2A), LDL (24.33%, 51.78%, and 47.13%, respectively; Fig. 2B), and triglycerides (39.67%, 40.47%, and 56.62%, respectively; Fig. 2C). The total cholesterol, LDL, and triglycerides in the group treated with simvastatin decreased by 27.96%, 44.69%, and 51%, respectively. All lipid parameters in the rats treated with the vehicle only significantly increased by 38.65%, 30.53%, and 17.23%, respectively.

6. Proinflammatory cytokine levels

The measurements of IL-6, TNF-α, and NFκβ p65 showed that the levels of these cytokines except NFκβ p65 in the group receiving the 200 mg/kg dose were significantly lower in the serum from the rats receiving extracts at 200, 300, and 600 mg/kg. The levels of IL-6 decreased by 34%, 36%, and 47%, respectively (Fig. 3A). TNF-α concentrations were decreased by 69%, 69%, and 70%, respectively (Fig. 3B). Upon treatment with 300 and 600 mg/kg extract, the NFκβ p65 concentrations were lowered by 35.6% and 35%, respectively (Fig. 3C).

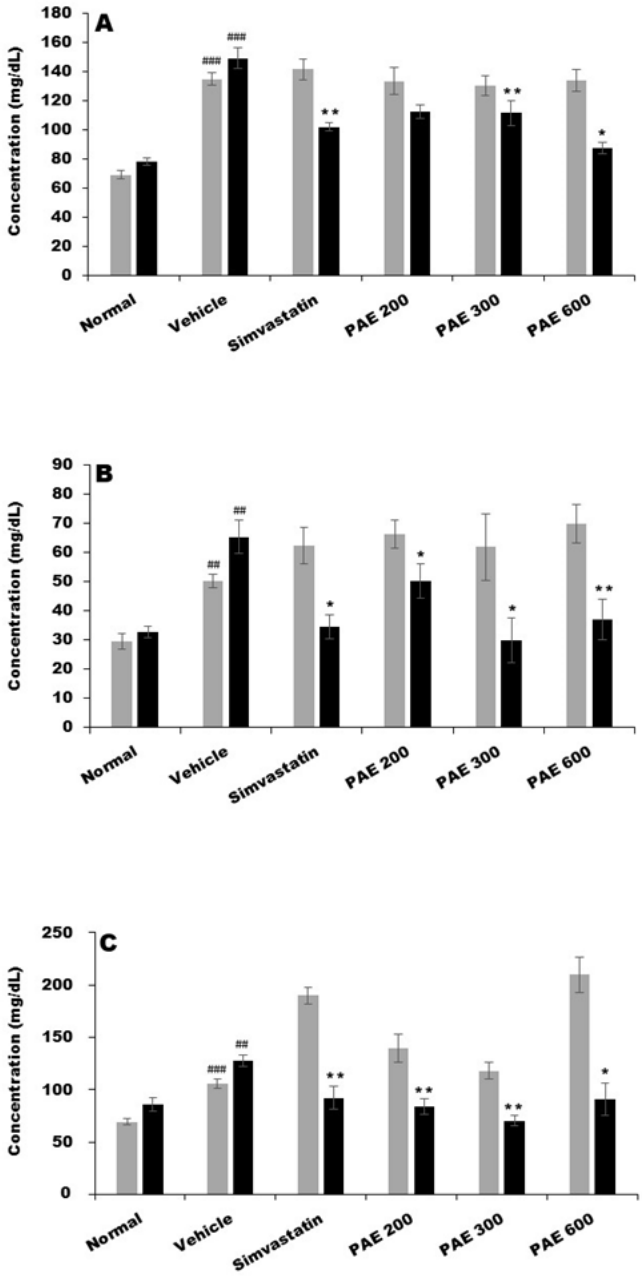

Figure 2. The effects of pandan leaves extract (PAE) on lipid parameters (total cholesterol, A; LDL, B; triglyceride, C) in a rat dyslipidemic model. The dyslipidemia was induced by administering high-fat feed for 8 weeks. Upon completion of the induction, the treatments were given for 4 weeks. Gray and black bars indicate pre- and post-treatment levels, respectively. Data represents average ± SEM of 3-7 rats. *, **, ***p < 0.05, 0.01 and 0.001 vs pre-treatment, paired t-test; ##, ###p < 0.01, 0.001 vs normal, unpaired t-test.

DISCUSSION

Dyslipidemia is closely related to obesity and coronary heart disease. Consuming high-fat food is one of the factors that cause increased fat accumulation, resulting in metabolic syndromes, such as dyslipidemia [16]. In this study, an animal model induced by high-fat feed was used since it has been widely utilized to represent human metabolic disorders [17]. The results showed that the administration of pandan leaf ethanol extract for 4 weeks significantly reduced total cholesterol, LDL, and triglycerides. The post-treatment levels of proinflammatory cytokines were also decreased.

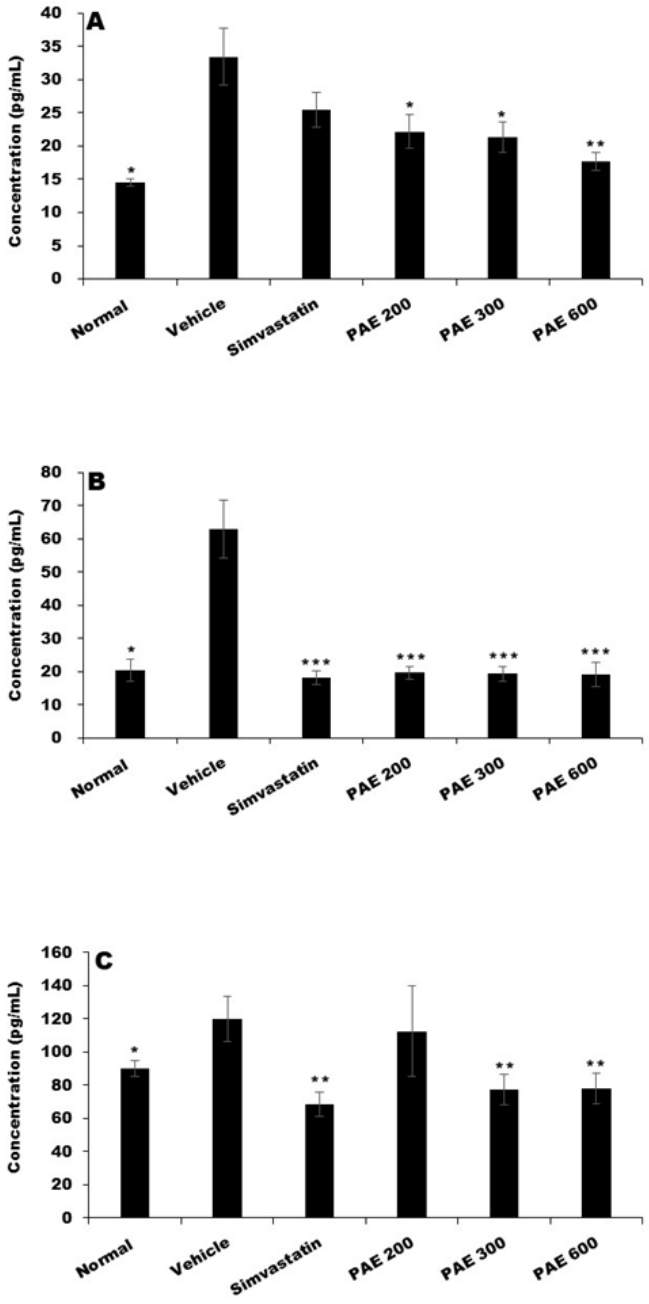

Figure 3. The effects of pandan leaves extract (PAE) on proinflammatory cytokines (IL-6, A; TNFalfa, B; NFκβ p65, C) in a rat dyslipidemic model. The dyslipidemia was induced by administering high-fat feed for 8 weeks. Upon completion of the induction, the treatments were given for 4 weeks. Data represents average ± SEM of 3-4 rats. *, **, ***p < 0.05, 0.01 and 0.001 vs vehicle, ANOVA *post hoc* PLSD.

Previous studies showed that pandan contains several metabolites, including alkaloids, flavonoids, and polyphenols. Consistent with previous findings, the results of the phytochemical screening of pandan ethanol extract revealed the presence of alkaloids, flavonoids, polyphenols, saponins, steroids, and triterpenes [18, 19]. LC-MS analysis further showed the presence of the alkaloid pandamarilactonine-A and B, as indicated by the component with an *m*/*z* of 318.17. Indeed, our recent *in silico* study demonstrated that this alkaloid actively interacts with HMG-CoA reductase, PPAR alpha, and NPC1L1 [13]. The mass spectrogram also revealed the presence of quercetin at *m*/*z* of 303.051. This flavonoid exhibits an antidyslipemic activity in animal models [20] and patients [21].

Phenolics and flavonoids are major groups of plant secondary metabolites with beneficial effects on health [22-24]. They scavenge free radicals and decrease oxidative stress. As such, they have gained interests in the fields of pharmacology and their clinical application. Phenolic compound production is commonly associated with stressors exposed to plants [23, 25]. In most cases, the abundance of plant secondary metabolites is in the following order: phenolics > alkaloids > flavonoids > terpenoids [26]. The total phenol and flavonoid contents of the extract were 31.76 mg/g QE and 80.91 mg/g GAE, respectively; as such, the extract was considered to contain moderate amounts of these metabolites. The phenol and flavonoid contents were dependent on the solvent polarity used in extraction [27].

IC_{50} of pandan leaf ethanol extract was 3.159 μg/mL, which is much greater than that of pravastatin (0.072 μg/mL). However, in comparison with other plants such as bay leaf (IC_{50} of 22.74 μg/mL) with inhibitory activities against the HMG-CoA reductase [28], pandan leaf extract might have a greater potential for development as a therapeutic agent in dyslipidemia treatment.

The *in vivo* activity test of pandan leaf ethanol extract showed that the extract at all doses reduced the blood concentrations of total cholesterol, LDL and triglyceride. Earlier studies revealed that phytochemical components reduce serum lipid levels through several mechanisms, such as inhibiting cholesterol absorption in enterocytes, reducing cholesterol synthesis, increasing reverse cholesterol transport, and increasing cholesterol excretion in the liver [29]. Our recent *in silico* study demonstrated that several alkaloids in pandan have potencies to exert antidyslipidemic effects. Thus, pandamarilactonine-A and B, pandanamine, and pandanusine B likely inhibit HMG-CoA reductase, activate PPAR alpha, and interfere with cholesterol transport via NPC1L1 [13]. Strobel and colleagues [30] found that flavonoid compounds such as quercetin and myrcetin prevent cholesterol synthesis by inhibiting the HMG-CoA reductase. In our present study, LC-MS characterization suggested that quercetin was a major chemical component.

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Dyslipidemia is one of the main factors related to atherosclerosis; in atherosclerosis, an atherosclerotic plaque is associated with a chronic inflammatory process that involves endothelial dysfunction and increased blood cholesterol levels. This process promotes the translocation of transcription factors that encode proinflammatory cytokine genes; it also inhibits the production of anti-inflammatory cytokines in macrophages [31].

TNF-α activates NFκβ p65 transcription and consequently regulates the expression of genes involved in inflammation, oxidative stress, and endothelial dysfunction. Its pro-atherogenic effects on the endothelium involves various mechanisms, including induction of reactive oxygen species production, decreased nitric oxide bioavailability, and increased endothelial permeability to circulating blood components and cells. TNF-α also regulates blood vessel permeability to control inflammation. When microvessel permeability increases, blood macromolecules and inflammatory cells can enter an injured tissue. Through this increased microvessel permeability, blood lipids and subendothelial inflammatory molecules accumulate, and atherosclerotic plaque forms [32, 33].

IL-6 may elicit pro-atherogenic and anti-atherogenic effects on processes associated with atherosclerosis development and progression [34]. Its pro-atherogenic effects include the activation of endothelial cells, stimulation of vascular smooth muscle proliferation, and activation of platelets. Its anti-atherogenic effect involves decreasing plasma LDL by increasing the gene expression of the LDL receptor. IL-6 mediates the acute phase response, thereby increasing the levels of reactants such as C-reactive protein, fibrinogen, and plasminogen activator inhibitor involved in the formation of atherosclerotic thrombosis [35-37]. It also promotes blood clot formation and vascular occlusion in atherothrombosis by inducing the aggregation and activation of platelets. This proposed mechanism likely involves an interaction with the microRNA miR-338-5p [38].

The present results showed that the levels of these cytokines, except NFκβ p65 in the group treated with 200 mg/kg of the extract, were significantly lower in the serum from the rats treated with 200, 300, and 600 mg/kg extracts. Previous results demonstrated the ameliorating effects of the metabolites of pandan on proinflammatory cytokines. For example, polyphenols reduce proinflammatory cytokine surge [39], and flavonoids decrease the cytokine content [40]. Therefore, the extract elicits a preventive effect against atherosclerosis and blockages in blood vessels. It may also have the potency to reduce the risk of heart attacks.

CONCLUSION

The ethanol extract of *P. amaryllifolius* Roxb. leaves has a potential antidyslipidemic activity. It can reduce the concentrations of IL-6, TNF-α, and NFκβ p65. These results further imply that this extract may be an alternative to treat dyslipidemia and prevent atherosclerosis.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest in this work.

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