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Antilipase and Antioxidant Activity of *Phyllanthus niruri* Methanolic Extract

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ABSTRACT

The aim of this study is to determine the antilipase and antioxidant activity of *Phyllanthus niruri* methanolic extract. The inhibitory activity against porcine pancreatic lipase (PPL) was determined by measuring the hydrolysis of *p*-nitrophenyl butyrate to *p*-nitrophenol at 405 nm. The crude extract exhibited strong lipase inhibitory activity with an IC_{50} value of 27. 65 µg/ mL. The inhibition mode study disclosed that *P. niruri* could act as noncompetitive inhibitor. Antioxidant activity of *P. niruri* extract was measured by 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay. *P. niruri* showed high antioxidant activity with an EC_{50} value of 7.471 µg/mL. The results suggested that *P. niruri* has shown potential as a source of natural antilipase and antioxidant.

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INTRODUCTION

Natural products provide a vast collection of pancreatic lipase inhibitors with potential for being developed into clinical products to treat obesity. Birari and Bhutani (Birari, R.B., K.K. Bhutani, 2007) reviewed a variety of plant extracts and secondary metabolites that have inhibitory activity against pancreatic lipase. The main phytochemicals contributed towards pancreatic lipase inhibition consisted of polyphenols, flavonoids, saponins and caffeine (Kim, H.Y., M.H. Kang, 2005; Han, L., *et al*., 2006; Moreno, D.A., *et al*., 2006; Shimoda, H., *et al*., 2006). On the other hand, consumption of edible vegetables with high antioxidant content is essential in combating degenerative diseases, such as coronary artery diseases, stroke, rheumatoid arthritis, diabetes and cancer (Harish, R., T. Shivanandappa, 2006). There is a great deal of interest in edible plants that contain antioxidants and health – promoting phytochemicals.

In this study, we aim to analyze the antilipase and antioxidant property of *Phyllanthus niruri* (family: Euphorbiaceae), a perennial herb distributed in tropical area. Previous studies reported that the whole plant, fresh leaves and fruits are used to treat various ailments, including hepatitis (Harish, R., T. Shivanandappa, 2006), antitumor and anticarcinogenic activities (Rajeshkumar, N.V., *et al*., 2002). Other medicinal properties, such as hypolipidaemic (Khanna, A.K., *et al*., 2002) and antiviral (Liu, S., *et al*., 2004) activities of *P. niruri* have also been shown.

MATERIALS AND METHODS

Preparation of Plant Extracts:

The whole plant material was air-dried and ground into fine powder. Hundred g of the powdered material was extracted by maceration in 1 L of 80 % methanol for 3 days at ambient temperature (25 – 30 ^{o}C). The extract was concentrated in a rotary vacuum evaporator at 45 ^{o}C and kept in a 4 ^{o}C chiller for long-term storage.

Lipase Preparation:

Porcine pancreatic lipase (PPL) was used as a model enzyme because of its high homology to human pancreatic lipase (85 % identity) and similar enzyme kinetics and behaviour (Lowe, M.E., *et al*., 1989). Crude lipase was dissolved in 50mM phosphate buffer pH 6 (1 mg/ml) and centrifuged at 12, 000 x *g* for 5 min to remove insoluble (Lewis, R., D. Direct, 2012; Lehner, R., R. Verger, 1997). Enzyme concentration was set at 0.1 mg/ ml. PPL, pNPB and crude extracts were freshly prepared prior to enzyme assay.

Lipase Inhibition Assay:

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The inhibition towards porcine pancreatic lipase was performed according to the modified method by Lewis and Liu (Lewis, R., D. Direct, 2012). The lipase activity was quantified by calculating the conversion of *p*-nitrophenyl butyrate to *p*-nitrophenol at 405 nm using UV-transparent 96-well plates on a microplate reader (BIO-TEK, Synergy HT, U.S.A.). Lipase assays were performed by incubating the plant extracts (final concentration 500 µg/ml) with PPL and pNPB in reaction buffer (50 mM potassium phosphate buffer, pH 7.2, 0.5 % Triton X-100) for 15 min. pNPB was first solubilised with dimethylsulfoxide (DMSO), then diluted with the reaction buffer to a final concentration of 2.5 mM in a 100 µl reaction. The concentration of DMSO in the reaction (1 %) did not affect the enzyme activity. All assays were run at 37 ^{o}C and the results are the average of three replicates that were blank subtracted. Orlistat was used as a positive control. The activity was examined with and without the inhibitor. Inhibition of the lipase activity is defined as the decrease in percentage of activity when PPL is incubated with the crude extracts. Lipase inhibition (%) is determined by using the formula:

Inhibitory activity (I %) = 100 – [(B − b)/ (A − a) × 100]

Where, A = the activity without inhibitor, a = the negative control without inhibitor, B = the activity with inhibitor and b = the negative control with an inhibitor. The concentrations of extracts, giving 50 % lipase inhibition (IC_{50}) were calculated from the least squares regression line of the semi-logarithmic plot against percentage inhibition curves using the GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, USA).

Kinetic Study:

The inhibition mode was determined by Hanes-Woolf plot analysis resulted from the enzyme assay data containing increasing concentrations of pNPB (0.25, 0.5, 1.0, 2.0, 4.0 and 6.0 mM) with the absence and presence of difference concentration of extract (10 and 50 µg/ml). All samples were analyzed in triplicate. Hanes-Woolf plot was constructed by plotting the ratio of the substrate concentration to the reaction velocity ([S]/v) against the substrate concentration [S]. Hanes-Woolf plot and nonlinear regression analysis for determination of Michaelis–Menten constant, K_m and maximal velocity, V_{max} were done using GraphPad Prism 4.0 software.

DPPH Free Radical Scavenging Activity Assay:

20 µl of methanolic 2,2-diphenyl-1-picrylhydrazil solution was added to 100 µl of sample solution in 96 well plates. The decrease in absorbance was determined at 515 nm using a microplate reader (BIO-TEK, Synergy HT, U.S.A.) after 30 minutes incubation period at room temperature. The test of each sample and ascorbic acid were conducted in triplicate (n = 3). Mean, standard deviation and IC_{50} of the results obtained were determined using GraphPad Prism software (GraphPad, USA). The percentage of the inhibition was calculated using the formula:

Scavenging Percentage % = 100-((AS/AC) x 100)

Where, AS = Absorbance of sample, AC = Absorbance of negative control (DMSO without sample)

All samples and readings were prepared in triplicate. The IC_{50} value of the extract was calculated from the plotted graph of scavenging activity against sample concentration using GraphPad Prism 4.0 software.

RESULTS AND DISCUSSION

Lipase-Inhibitory Activity:

Preliminary assay revealed that *P. niruri* extract inhibited 76.7% pancreatic lipase activity when incubated with PPL at a final concentration of 500 µg/ml for 15 minutes at 37^{o}C. The findings from IC_{50} value showed that the *P. niruri* extract markedly inhibited the pancreatic lipase activity with the IC_{50} value of 27.7 µg/ml. However, the extract was less potent than orlistat (control) in inhibiting pancreatic lipase. Orlistat gave an IC_{50} value of 1.7 µg/ml, which was about 17 times stronger than *P. niruri* crude extract (Table 1). This is predictable since the crude extract possesses both active and non-active compounds which may affect the effectiveness.

Table 1: PPL inhibitory activity of *P. niruri* methanolic extracts (80%).

Scientific name	Family	Common name	Part used	Inhibition (%)[a]	IC_{50} (µg/ml)[b]
Phyllanthus niruri	Phyllanthaceae	Dukung anak	Whole plant	76.7 + 0.4	27. 7
Orlistat (Control)				99.6 + 0.3[c]	1.7

[a] Percentage of inhibition represents the ability of extract to inhibit pancreatic lipase activity in the medium; concentration tested was 500 µg/ml in the assay except for [c]. Determined base on the average of three independent replications.
[b] IC_{50}, concentration causing 50% inhibition; concentration tested were varies from 10^{-2} to 10^{3} µg/ml in the assay.
[c] Concentration tested was 10 µg/ml in the assay.
n = no inhibition
nd = not determined

Inhibition Mode:

The inhibition mode of *P. niruri* extract was visualized using graphical representation of the Michaelis–Menten equation, Hanes-Woolf plot; [S]/v versus [S] as shown in Figure 1. The enzyme kinetic result disclosed that *P. niruri* exerted an inhibitory effect on pancreatic lipase in a noncompetitive manner. As depicted from the graph, when the

extract concentration was increased, the value for the *y*-intercept in the equation for each curve increased, whereas the *x*-intercept remained at fixed point showing these inhibitors do not affect K_m but the V_{max} decreased.

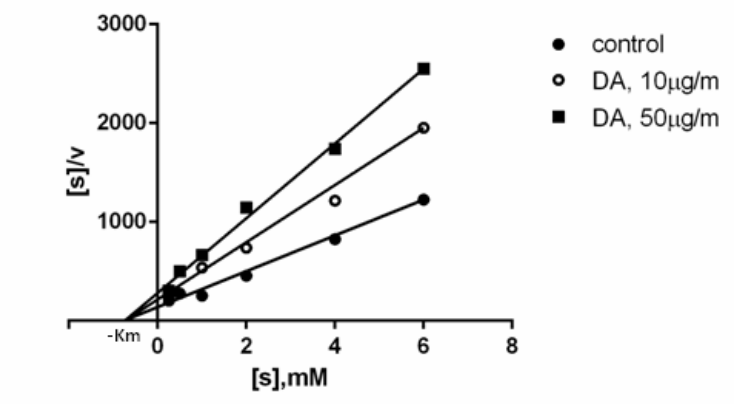

Fig. 1: Inhibition mode determination. Hanes-Woolf plot; [S]/v versus [S] of kinetic analysis for PPL at 2 different concentrations of *P. niruri* extract (Abbreviated as DA = dukung anak).

Free radical scavenging activity test:

The potential of *P. niruri* extract as a free radical scavenger was screened using the DPPH scavenging assay. As depicted in Figure 2, the DPPH free radical - scavenging activity of *P. niruri* extract as well as positive control is concentration dependent. The antioxidant activities of the *P. niruri* extract increased consistently with the concentration. *P. niruri* extract showed great ability to inhibit DPPH radical with recorded EC_{50} value of 7.471 µg/ml. Ascorbic acid was served as the positive control with the estimated EC50 of 2.628 µg/ml.

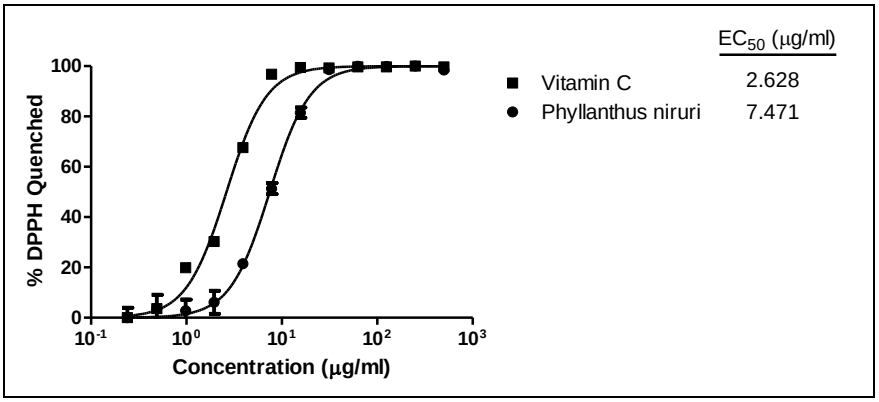

Fig. 2: Antioxidant activity of *P. niruri* extract and positive control at different concentration defined as inhibition percentage of DPPH● in DPPH assay. Values are mean ± standard deviation (n = 3). Ascorbic acid (Vitamin C) was used as positive control.

Conclusions:

In this paper, we have determined high antilipase and antioxidant activities of *P. niruri* plant extract. The results provide preliminary indicator in our searches for the responsible compounds that contribute to the antilipase and antioxidant activities.

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