

Antioxidants Properties of *Phyllanthus niruri* (Dukung anak) Extracts

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ABSTRACT The antioxidant activity of *Phyllanthus niruri* (dukung anak) extracts (methanol, water, chloroform), and fractions (ethyl acetate, n-butanol) was evaluated by various antioxidant assays, including total antioxidants, free radical scavenging, superoxide anion radical scavenging, and β -carotene bleaching antioxidant capacity. These various antioxidants activities were compared to standard antioxidant, butylated hydroxytoluene (BHT) and quercetin. Xanthine oxidase (EC 1.1.3.22) inhibition activity of the extracts was also evaluated. The extracts and fractions showed varying degrees of antioxidant activity through all assays. The chloroform extracts exerted a significant activity in the β -carotene assay, but no activity in free radical and superoxide anions scavenging were noted. It also showed weak inhibition of xanthine oxidase. The antioxidant property is concentration dependent. The results obtained in the present study indicated *P. niruri* (dukung anak) leaf extracts are a potential source of natural antioxidants.

Keywords: *Phyllanthus niruri*; leaf extracts, fractions; antioxidant activity.

INTRODUCTION

P. niruri, a small plant which grows mainly in tropical and subtropical regions in Central and South American countries, India, and East Asia, is one of the most important medicinal plants used by people in these countries for treatment of jaundice, asthma, hepatitis, urolithic disease, fever, malaria, stomachache and tuberculosis [1]. Extensive chemical examinations of this plant have been carried out and several constituents were isolated such as lignans, alkaloids, flavonoids, tannins, phthalic acid, gallic acid and terpenoids [2, 3].

Many antioxidants compounds from plant sources have been identified as free radical or active oxygen scavengers. Recently, interest has increases considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, which are being restricted due to their side affected such as carcinogenicity [4]. Natural antioxidants can protect the human body from free radicals and the progress of many chronic diseases as well as retard lipid oxidative rancidity in foods [5-8].

Reactive oxygen species (ROS) including free radicals such as superoxide anion radicals ($O^{\cdot -}$),

hydroxyl radicals (OH^{\cdot}), and singlet oxygen (1O_2) are various forms of activated oxygen and often generated by oxidation product of biological reactions or exogenous factor [9-11].

ROS have aroused significant interest among scientists in the past decade. Their broad range of effects in biological and medicinal systems has drawn on the attention of many experimental works. ROS can cause lipid peroxidation in foods, which leads to deterioration of the food [12].

In this paper, we describe the antioxidant activity of *P. niruri* (dukung anak) extracts and fractions which was evaluated by various antioxidant assays to cover most of the possible mechanisms of oxidation reactions.

MATERIALS AND METHOD

Chemicals

(Tween-20), 1,1 Diphenyl-2-picryl-hydrazyl (DPPH), Nicotinamide adenine denucleotide (NADH), Butylated hydroxytoluene (BHT), β -carotene, linoleic acid, xanthine, xanthine oxidase, rutin and allopurinol were purchased from Sigma Chemical Co. All other chemicals and reagents were analytical grade.

Plant material and extraction

The leaves of *P. niruri* were dried by oven and it was milled into powder. Ten grams each of powdered leaf samples were extracted separately with 100 ml chloroform, methanol and water at 70° C for two hours. The methanol extract was dissolved in water and successively extracted with ethyl acetate, n- butanol and water. The solvents were evaporated to dryness and the residues dissolved in methanol or dimethylsulfoxide (DMSO) to prepare the desired concentrations.

β-carotene assay

The method was adapted from [13] with some modifications. Linoleic acid (0.02 ml) and tween20 (0.2 ml) were placed in (100 ml) round flask, and (1ml) β-carotene (0.2 mg/ml in chloroform) was added to the flask. The mixture was evaporated to dryness under vacuum in the dark. Fifty milliliter of distilled water was added to mixture and shaken. 3.8 ml of the mixture was then dosed with 0.2 ml of corresponding concentration of tested sample or reference compound and the final concentrations (50µg/ml), BHT and quercetin 0.25 µg/ml) was used as reference compound. Sample without dose was used as control.

The absorbance was read on spectrophotometer at 470 nm. The samples were then subjected to auto oxidation, thermally-induced with constant water bath temperature at 45°C for 2h. Absorbances were taken at 15 min intervals to monitor the rate of bleaching of β- carotene (triplicate). On the basis of the bleaching rates, three parameters relative to control were calculated in order to compare the antioxidant abilities of the extracts. The calculations of all three parameters were deemed to be necessary for comparison because similar data were available in the literature [13]. Antioxidant activity (AA) was determined as percentage inhibition relative to control sample using the following equation;

$$A_A = ((R_{control} - R_{sample}) / (R_{control})) \times 100 \text{ ----- (a)}$$

where $R_{control}$ and R_{sample} represent the bleaching rates of β-carotene without and with the addition of antioxidant, respectively.

$$\text{Degradation rates } RD = \ln (A_i / A_x) \times 1/t \text{ ----- (b)}$$

Where \ln is natural log, (A_i) is the initial A_{470} ($t=0$), and A_x is the A_{470} at $t=10, 20, 30$, etc.min. The oxidation rate ratio (R_{OR}) was calculated using the equation;

$$R_{OR} = R_{sample} / R_{control} \text{ ----- (c)}$$

Where R_{sample} and $R_{control}$ are as described previously.

The antioxidant coefficient (C_{AA}) was calculated using the following equation

$$C_{AA} = (A_{s(120)} - A_{c(120)}) / A_{c(0)} - A_{c(120)} \times 1000 \text{ ----- (d)}$$

Where $A_{s(120)}$ is the absorbance of the sample containing antioxidant at $t=120$ min, $A_{c(120)}$ is the absorbance of the control at $t=120$ min, and $A_{c(0)}$ the absorbance of the control at $t=0, 15, 20$

Free radical scavenging activity (DPPH Assay)

The scavenging activity of DPPH free radical of *P. niruri* plant extracts was done according to the method reported by [5] with some modifications. The extract dissolved in methanol, 200 µl of each extract was mixed with 4 ml of 0.1 mM DPPH-methanol solution. The final concentrations were 6.125, 12.5, 25, and 50 µg/mL. Methanol was used as blank for this experiment. After 60 min of incubation at room temperature, the reduction of the DPPH free radical was measured at 517 nm. The lower absorbance indicated higher free radical scavenging activity. BHT was used as positive control.

Superoxide anions scavenging activity

Superoxid anions scavenging activity were estimated by the spectrophotometric measurement of the product of the reduction of nitro blue tetrazolium NBT [5, 14]. Superoxide anions were generated in a non-enzymic system (phenazine methosulphate-NADH). The non-enzymic generation of superoxide anions was measured in samples which contained 10 µM phenazine methosulphate, 78 µM NADH and 25µM NBT in 0.1M phosphate buffer pH 7.4. After 5 min of incubation at room temperature the colour was measured at 560 nm against blank sample, which contained no phenazine methosulphate. The inhibition ratio (%) was calculated from the following equation; Inhibition % = [(Absorbance control - Absorbance of test sample) / (Absorbance control)] × 100.

Xanthine oxidase inhibition

Xanthine oxidase (EC 1.1.3.22) inhibition activity was also evaluated by spectrophotometric measurement of the formation of uric acid from exanthine [14]. 100 µM solution of xanthine in 0.1M phosphate buffer pH 7.8 with 0.1 units/ml of xanthine oxidase was incubated for 10 min at room temperature and measured at 295 nm against a blank sample which did not contain the

enzyme. Various concentrations of tested samples and chemical inhibitor allopurinol were added to samples before the enzyme and had been instilled and their effect on the generation of uric acid was calculated from the equation;
Inhibition% = $(1 - A_s/A_c) \times 100$

Total phenolics

The method used for the determination of total phenols using Folin Ciocalteu reagent was adapted from [15]. Diluted extract (0.1ml of 1mg/ml in methanol) or phenolic standard was mixed with Folin Ciocalteu reagent (3 ml, 1:10 diluted with water) and aqueous Na_2CO_3 (3 ml, 1 M). Solutions were heated in a water bath at 45°C for 15 min. and the total phenols were determined colorimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg/l solutions of gallic acid in methanol. Total phenol values were calculated from the equation of the calibration curve and expressed as gallic acid percentage, which is a common reference compound.

Statistical analysis

Experimental results were means \pm S.D of two or three measurements. Differences between means were analyzed using ANOVA one-way program. P values <0.05 were regarded as significant and P <0.01 very significant.

RESULT AND DISCUSSION

β -carotene bleaching activity test used to assess potency of the extracts of *P.niruri* as antioxidants is a well established model system based on β -carotene assay coupled reaction with autoxidized linoleic acid. There is a gradually decrease in A 470 nm with β -carotene bleaching. Based on this testing with reference compound BHT, the concentration of which produced measurable inhibition in β -carotene bleaching. On the basis of the bleaching rates three parameters relative to control were calculated in order to compare the antioxidant abilities of the extracts. The polar extracts and fractions showed varying degrees of antioxidant activity through all parameters. Table.1 shows the results of antioxidant activity.

The order of activity of the tested samples according to antioxidant activity value (A_A) was $\text{CHCl}_3 > n\text{-BuOH} > \text{EtOAc} > \text{quercetin} > \text{MeOH} > \text{Water} > \text{BHT}$. However on the basis of oxidation rate ratio (R_{OR}) the order was found as following

$\text{BHT} > \text{Quercetin} > n\text{-BuOH} > \text{EtOAc} > \text{CHCl}_3 > \text{MeOH} > \text{Water}$. Higher value of R_{OR} indicated less activity. Antioxidant activity coefficient (C_{AA}) values of the samples in contrast shifted the order of all tested samples as follows: $\text{EtOAc} > n\text{-BuOH} \ \& \ \text{CHCl}_3 > \text{MeOH} > \text{BHT} > \text{Water} > \text{Quercetin}$.

All the extracts and fractions were exhibited very significant difference when compared with the control ($p < 0.01$) sample, which contains no antioxidant (Fig.1).

Free radical scavenging activity (DPPH assay).

The free radical scavenging activity of *P.niruri* extracts was assessed by DPPH assay. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [16]. The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. Hence, DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [17]. As shown in (Fig .2) all extracts except chloroform extract demonstrated concentration dependent inhibitory activity against the DPPH radical comparing to that of BHT and quercetin which are well-known antioxidant compounds and no significant difference between the positive control BHT ($P > 0.05$) and the samples activities. However there was no significant difference between the methanol and water extracts ($P > 0.05$). The complete inhibition by tested samples was observed at range of 12.5-50 $\mu\text{g/ml}$.

These results indicated that the *P.niruri* extracts (methanol, water) and fractions (ethyl acetate, n-butanol) have a noticeable effect on scavenging free radicals.

Superoxide anions scavenging activity

In the PMS/NADH-NBT system (non-enzymic method), superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 556 nm with antioxidants thus indicated the consumption of superoxide anion in the the reaction mixture (antioxidant). In this experiment both methanolic and aqueous extract and quercetin inhibited the reduction of NBT, whereas chloroform extract and BHT were not active (Fig.3).

Xanthine oxidase inhibition

In the xanthine-xanthine oxidase system the methanol and water extract showed inhibition percentage through all the used concentrations comparable with that of the chemical inhibitor (drug) allopurinol (Fig.4). The methanol extract exerted very significant inhibition ($P < 0.05$) compared with allopurinol. The xanthine oxidase inhibition activity of the methanol was higher than that of the water extract at concentrations of 10, 20 $\mu\text{g/ml}$, but there was no significant difference ($P > 0.05$) between the methanol and water extracts at concentration of 30 $\mu\text{g/ml}$. The activities of the extracts and allopurinol were concentration dependent. Xanthine oxidase inhibition activity of the methanol and water extracts support the traditional belief that the leaves of *P. niruri* may improve kidney stone disease. It's well known that approximately 10% of patients with kidney stone disease develop uric acid stones. Which are more common in men. The digestion of protein produces uric acid. If the acid level in the urine is high or too much acid is excreted, the uric acid may not dissolve and uric acid stones may form [18].

Total phenolic

Total phenolic compounds are very important plant constituents because of their scavenging ability due to their hydroxyl groups [19]. The content of phenolic compounds of the extracts and fractions of *P. niruri* was examined based on Folin-Ciocalteu assay. The results showed

measurable amount of phenolics in the polar extracts and fractions (Table.2). The effectiveness of the antioxidants of the tested plant extracts could be related to the phenolic content. It is proposed here that phenolic compounds of the *P. niruri* extracts may play an important role in the observed antioxidant activities and other medicinal benefits of this plant.

CONCLUSION

The water and methanol extract of *P. niruri* (dukung anak), showed strong antioxidant activity in β -carotene bleaching, DPPH radical, and superoxide anion scavenging and xanthine oxidase inhibition when compared to standards. There is clear relationship between the total phenol contents of the samples and their antioxidant activities. The lack of activity of the chloroform extract may be attributed to the low percentage of phenolic in the extract in addition to its lipophilic character. The result of this study show that the water and methanol extract of *P. niruri* can be used as easily accessible source of natural antioxidants in food supplement or in pharmaceutical industries. Therefore, it is suggested that further work could be performed on the isolation and identification of antioxidants in this herb.

Table 1. Parameters Used to Assess the Antioxidant Capacity of the Samples Tasted.

Sample	A_A^b	R_{OR}^c	C_{AA}^d
Control	0.00*	1.00	0.00
methanol extract	58.60	.41	494.50
Water extract	58.00	0.42	464.90
ethyl acetate fraction	68.50	0.32	546.10
n-butanol fraction	68.80	0.30	535.00
Quercetin	62.40	0.29	347.00
BHT	56.80	0.29	465.00
Chloroform extract	71.40	0.38	535.00

Note: ^b Antioxidant activity. ^c Oxidation rate ratio. ^d Antioxidant activity coefficient)

Table 2. Total Phenolic content (gallic acid %) of dry weight extract

Sample	Total phenolic %
methanol extract	34.09 \pm 2.84
water extract	33.50 \pm 1.66
ethyl acetate fraction	55.38 \pm 1.66
n-butanol fraction	45.01 \pm 5.03
chloroform extract	9.04 \pm 1.43

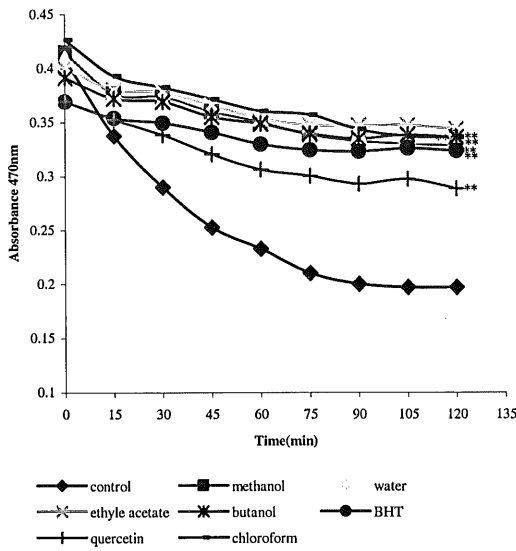


Figure 1. β -carotene assay of *P. niruri* extracts (50 μ g/ml) values are mean \pm SD, (n=3) **P<0.01

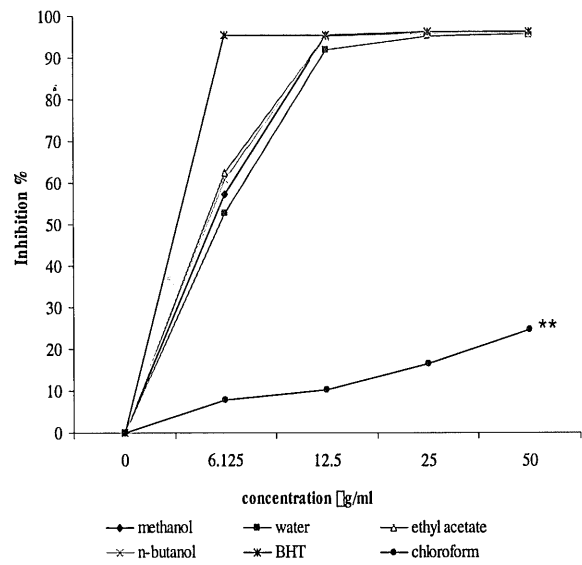


Figure 2. DPPH assay of *P. niruri* extracts. Results are mean \pm SD (n=3) **P < 0.01

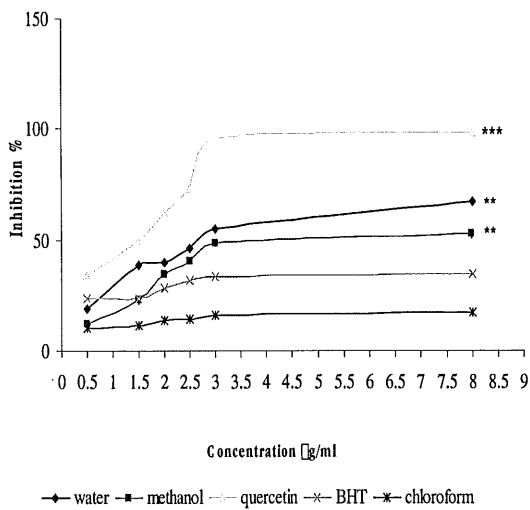


Figure 3. Antioxidant activity of *P. niruri* extracts as scavengers of superoxide anion Results are mean \pm SD (n=3) **P<0.01, ***P<0.001

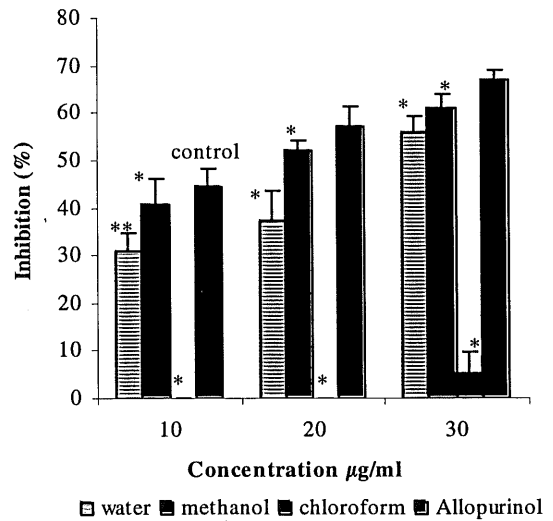


Figure 4. Xanthine oxidase inhibition activity of extracts. Results are mean \pm SD (n=3) *P<0.05, **P < 0.01

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