Antioxidant activity of *Phyllanthus niruri* L. herbs: in vitro and in vivo models and isolation of active compound

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**ABSTRACT**

**Background:** *Phyllanthus niruri*, *Thymus vulgaris*, *Centella asiatica*, and *Apium graveolens* L. have been frequently used as medical herbs for various diseases. However, the use of the herbs is not based on any evidence of their contents. **Aims and Objective:** To examine their potential activity of becoming antioxidant, using in vitro and in vivo models and isolate active compound. **Materials and Methods:** The antioxidant activity of the samples was assessed using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as radical compounds. The decrease of DPPH radical was measured using spectrophotometer at 517 nm, after an incubation for 30 min. The highest antioxidant activity of the sample was then continued with in vivo model using Sprague Dawley (SD) rats. Paracetamol was used as an inducer to increase the malondialdehyde (MDA) levels in plasma, with doses of 2.5 g/kg BW. The rats were divided into four groups: negative control, dose of 50 mg/kg BW, dose of 100 mg/kg BW, and dose of 200 mg/kg BW. Isolation method was guided by antioxidant activity by using fractionation and column chromatography. The obtained compound was then characterized by 1H-nuclear magnetic resonance (NMR) and compared with the earlier study. **Result:** The IC50 value of these samples were 14.21 ± 0.73; 14.41 ± 1.13; 98.66 ± 9.59; and 237.33 ± 17.26 µg/mL, respectively, and the total phenolic contents were 81.59 ± 2.85; 154.09 ± 11.61; 6.36 ± 3.99; 2.03 ± 0.78 mg/g of sample extract, respectively. The in vivo study showed that *P. niruri* with dose of 100 mg/kg BW and 200 mg/kg BW were able to reduce MDA levels by 90.44% and 92.64%, respectively. Meanwhile, the proton and carbon NMR spectra showed that the isolate was quercetin. The IC50 value of the isolate, being 5.85 µg/mL, was lower than that of vitamin E, which was 6.85 µg/mL. **Conclusion:** The extract shows a potential effect with antioxidant activity, both in in vitro and in vivo models. Quercetin is the active compound, the antioxidant activity of which is higher than that of vitamin E.

**KEY WORDS:** Antioxidant; *Phyllanthus niruri* L; Quercetin

**INTRODUCTION**

Free radicals have one or more electrons that are not paired in the outer orbitals[1] and produced under normal circumstances of metabolic processes.[2] Free radicals can damage proteins, lipids, carbohydrates, and nucleic acids.[3] Over longer terms, some damages can lead to degenerative diseases.[4] In diabetic patients, for example, hyperglycemia causes glucose auto oxidation, glycation of proteins, and activation of the polyol metabolic pathway that will accelerate the formation of reactive oxygen species (ROS).[5] This will result in an imbalance between antioxidants in the body and free radicals, which is the beginning of oxidative damage, known as oxidative stress. The risk of the disease can be minimized by the use of antioxidant compounds that can stabilize radicals.[6,7]

Obviously, the sources of antioxidants are natural and synthetic antioxidants. The Indonesian people have used a variety of medicinal plants to heal various diseases. A previous
study on 23 medicinal plants in Indonesia found four plants (*Phyllanthus niruri*, *Thymus vulgaris*, *Centella asiatica*, and *Apium graveolens*) that are potential as antioxidant agents, the activity of which is better or equal to that of vitamin E. These plants contain alkaloids, flavonoids, lignin, tannins, coumarin, polyphenols, saponins, and terpenoids. Active compounds that may work as antioxidants are tannins and alkaloids and phenolic components, such as phenolic diterpene, phenolic acids, and flavonoids. Owing to reducing properties, the antioxidant activity of the phenolic content can stabilize singlet oxygen, capture free radicals, electron donors, and chelating metals.

Therefore, this study was conducted to explore the potential of antioxidants from those four medicinal plants that have the best antioxidant activity based on in vitro and in vivo models, isolate the active compounds, and characterize the structure by proton and carbon nuclear magnetic resonance (NMR). The antioxidant activity is generally contributed by the phenolic content of the extract. The isolate was assessed through antioxidant activity and compared with vitamin E as an established antioxidant agent.

### Materials and Methods

The laboratory equipment used for this study was glassware (Pyrex), analytical balance (Ohaus), minispin (Eppendorf), centrifuge (Centrifuge PLC Series), Spectrophotometer uv-vis (Shimadzu 1140), ultrasound (Branson), and NMR (JEOL).

The materials used in this study consisted of *P. niruri* L., *T. vulgaris*, *C. asiatica*, and *A. graveolens* (local market), rats Sprague Dawley (SD) strain, paracetamol 2.5 g/kg BW (Pharmaceutical grade, Brataco Chemica), TMP-X (Sigma Aldrich), Vitamin E (Sigma Aldrich), and plate silica gel GF254. This study was approved by Health Research Ethics Committee of Faculty of Medicine, Universitas Muhammadiyah Surakarta.

### Extraction

The extraction process was performed by maceration using ethanol 96% as a solvent with a 1:7 ratio. Liquid extract was evaporated using a rotary evaporator and then heated in the water bath until a thick extract was formed.

### Determination of Antioxidant in In Vitro Model

Seven hundred μL of DPPH solution (400mM) plus 1.5 mL of sample solution of various concentrations were then left in the dark for 30 min and then read at a wavelength of 517 nm. The blank used was 1.5 mL sample solution added by ethanol up to 5.0 mL, while the control used was 700 μL DPPH solution added by ethanol up to 5.0 mL. Inhibition concentration (IC_{50}) was the concentration of extract or fraction that gave antioxidant activity of 50%.

### Determination of total phenolic contents

The total phenolic content was determined using the Folin–Ciocalteu reagent according to the method of Waterhouse. The reaction mixture consisted of 100 μL of extract and 100 μL Folin–Ciocalteu reagent. The mixture was kept in darkness for 5 min, then combined with 75 μL of sodium carbonate and made up to 5.0 mL of aquadest. In order to complete the reaction, the mixture was kept in darkness for 45 min at room temperature. The absorbance at 742.5 nm was measured using a UV-Vis spectrophotometer (Shimadzu Corp.). Gallic acid was used as a standard, and the result was expressed as gallic acid equivalent (GAE)/g extract.

### Determination of Antioxidant In Vivo Model

The hepatoprotective activity of *P. niruri* extract was determined with hepatotoxic rat model using paracetamol as the inducer. After 7 days of acclimatization, the rats were classified into four groups of five. The treatments were done for 8 days. Group I served as the normal control and received only aquabidest (0.4 mL/kg BW) for 8 days. The group II served as the toxic control and was administered with paracetamol suspension (2.5 g/kg BW, po) once in every 72 h. Groups III until V were administered with extract (50; 100; and 200 mg/kg BW, po) daily and paracetamol suspension (2.5 g/kg BW, po) once in 5 days. About 1 mL of blood was collected from vena lateralis of rats on days 0, 5, and 8. The blood samples were centrifuged at 3,000 rpm at room temperature to separate serum and used to estimate the MDA levels.

### Isolation and Characterization

Isolation of active compound of *P. niruri* was conducted by guiding antioxidant in in vitro models. The extract of *P. niruri* was fractionated to obtain n-hexene fraction, ethyl acetate fraction, and residues fraction. All fractions obtained were then assessed with antioxidant activity. Then, ethyl acetate fraction, which has a potential activity, was fractionated with vacuum column chromatography. Stationary and mobile phases used were silica gel (Merck 7734) and a mixture of CHCl₃-n-hexene (8:2) and CHCl₃-methanol (9:1, 8:2, 7:3, 6:4, and 5:5). Elution was performed three times with volume of each mobile phase of 350 mL. The volume of the collected fraction was 130 mL. Altogether, 58 fractions were obtained. On the basis of the thin
After TLC examination, nine subfractions were collected, and, then, the antioxidant activity was determined. The highest antioxidant activity of subfraction, then fractionated by flash chromatography, silica G 60 (Cat Number 7731) as stationary phase and mixtures of acetonitrile:methanol:water = 1:1:1 as mobile phase. Elution was performed three times, each using 200 mL. Fraction was collected; the separation profiles were then evaluated by TLC. The same profiles combined resulted in four subfractions, and, then, the antioxidant activity was assessed. Owing to the impurity content of subfraction, the purification was performed by preparative RP-TLC (Silica-C18, as a stationary phase and mixtures of acetonitrile:methanol:water = 1:1:1 as mobile phase). Separated subfraction was then cleaned up using flash chromatography [methanol:CHCl3 = 3:7, as mobile phase, and silica G 60 (Cat Number 7731), as stationary phase]. The isolate characterized by proton NMR (JEOL, 500 MHZ), CDCl3 was used as a solvent. Antioxidant activity of isolate was assessed and compared with vitamin E.

Result

Screening Antioxidant Activity
Potential antioxidant activity was classified based on the IC50 value.17 The result showed that the antioxidant activity of P. niruri and T. vulgaris showed similar values, around 14 μg/mL, which were close to that of the vitamin E (Table 1). The other extracts are not active as an antioxidant.

Antioxidant of In Vivo Models
The objective of in vivo models was to optimize the induction time of paracetamol as hepatotoxic inducer, which is administered per oral. Induction of hepatic may increase MDA levels in serum. As shown in Table 2, the optimum time to measure the MDA levels is 72 h after induction. The increase of MDA is almost double of the first time, about 62.88 ± 12.38 μg/mL.

Isolation and Characterization of Active Compound
The result shows that the rendemen of nonpolar fraction is the lowest, while residue is the highest (Figure 1). This indicates that the chemical components of the extract are mainly polar and semipolar compounds.

Characterization of Isolate 4 by proton NMR
The spectra of proton MNR show five peaks (Figure 3A). These peaks at 7.728 ppm (d,J = 2.6 Hz) represent proton position at 2’; 6.879 ppm (d,J = 10.60 Hz) represent proton at 5’; 7.617 ppm (d,J = 10.65 Hz, 2.65 Hz) represent proton at 6’; and 6.384 ppm (d,J = 2.5 Hz) represent proton at 6’; and 6.177 ppm (d,J = 2.5 Hz) represent proton position at 8’ (Figure 2).
and tannins. The differences of antioxidant activity can be the influence of several factors, such as the solvent of extraction, chemical composition of samples, and determination methods.[21] Evaluation of antioxidant of in vivo models was conducted by referring to the activity of previous study on antioxidant and antidiabetic effects of *P. niruri*. The data [Table 3] shows that *P. niruri* presents an antioxidant activity that is characterized by the decrease of MDA levels in the serum. The decrease of *P. niruri* dose of 100 and 200 mg/kg BW was almost close. This study also shows a similar result[23] that *P. niruri* presents an antioxidant

![Figure 1](image_url)  
Figure 1: Scheme for isolation of antioxidant compound from extract *P. niruri.*
activity using in vivo model. Chemical composition of *P. niruri*, such as transiphytol, 4-t-butylpyrocatechol, phyllanthin, and hypophyllantin can protect cytotoxicity hepatocyte culture.[24,25]

Isolation is guided by the antioxidant activity, initiated by redissolving the extract by using *n*-hexene (as nonpolar solvent), ethyl acetate (as semipolar solvent), and residue (as polar fraction). On the basis of the evaluation of the antioxidant, the candidate to be isolated was the ethyl acetate fraction. In order to obtain simple components in the fractions, gradient polarity mobile phase is used in the vacuum column chromatography. The fraction obtained classified based on the TLC profiles became nine subfractions. The assessment of antioxidant activity shows that group VI shows the highest activity [Figure 1]. On the basis of the results in Fig 1, the antioxidant activity of isolated compound are higher than vitamin E (IC50 = 6.85 μg/mL) which is known as an established antioxidant compound. Isolate 4 can be elucidated by using 1H-MR and compared with the reference. The spectra of isolate [Figure 3A] are quite similar to the result of an earlier study (Fig. 3B).[26] Therefore, the isolate is quercetin.

**CONCLUSION**

*P. niruri* L. herb is a promising antioxidant herb that has the best DPPH radical scavenging activity when compared with *T. vulgaris, C. asiatica*, and *Apium graveolens* L.; meanwhile, the extract at dose 200 mg/kg BW could decrease MDA levels around 92.64%. Isolation guided by the antioxidant activity of *P. niruri* as a promising antioxidant herb showed the fractions.

<table>
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<th>Table 3: MDA levels of groups treatments</th>
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<td><strong>Groups</strong></td>
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<td>Negative control</td>
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<td><em>P. niruri</em>, 50 mg/kg BW</td>
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<td><em>P. niruri</em>, 100 mg/kg BW</td>
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<td><em>P. niruri</em>, 200 mg/kg BW</td>
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ND = not detected.
*p < 0.05, compared with negative control group (one-way ANOVA, followed by a t-test)
of n-hexane, ethyl acetate, and ethanol, which showed values of IC50 such as 14.21 ± 0.73, 282.84 ± 11.77, 2.67 ± 0.74, and 8.85 ± 1.54 µg/mL, respectively. Quercetin was successfully isolated from the extract and showed a higher antioxidant activity than vitamin E.

References

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