RESEARCH ARTICLE

Antioxidant Activity of *Phyllanthus niruri* Extract, Rutin and Quercetin

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Abstract

BACKGROUND: Normal metabolism of oxygen and exogenous factors constantly generate free radicals which could be harmful to the human body. Human need antioxidants to provide protection against free radicals, thus plants are a good source of natural antioxidants. *Phyllanthus niruri* (*P. niruri*) has been known to possess several medicinal properties and contain numerous active phytochemical. In this research, we conducted phytochemical screening and antioxidant assay of *P. niruri* extract along with the compounds rutin and quercetin, which are flavonoids possessing medicinal properties. This study was conducted to determine *P. niruri*, rutin and quercetin as antioxidant.

METHODS: In this study, qualitative phytochemical screening was performed to detect phenol, flavonoid, saponin, tannin, steroid/triterpenoid, terpenoid and alkaloid in *P. niruri* extract. Antioxidant analysis of *P. niruri*, rutin and quercetin was conducted using total measured phenolic content, 2,2-diphenyl-1-picrylhydrazil (DPPH), 2,2'-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant power (FRAP) assays.

RESULTS: The study revealed that *P. niruri* extract contained saponin, phenol, flavonoid and tannin based on phytochemical screening. In DPPH and ABTS assays quercetin possessed highest antioxidant activity with IC_{50} value of 0.55 and 1.17 µg/ml respectively. Meanwhile, *P. niruri* extract showed the highest FRAP activity which was 373.95 µM Fe(II)/µg extract. Rutin possessed the lowest antioxidant activity in all antioxidant assays.

CONCLUSION: This study confirmed that *P. niruri* extract and quercetin have great potential as a natural antioxidant source.

KEYWORDS: asntioxidant, phytochemical, *Phyllanthus niruri*, quercetin, rutin, free radical

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Introduction

Reactive Oxygen Species (ROS) leads to damaging the lipids in the cell membranes, proteins in tissues as well as enzymes, carbohydrates, and DNA to induce oxidation. This oxidative damage may play a causative role in aging and several diseases which are cancer, cardiovascular disease, cataracts, and cognitive dysfunction.(1,2) The high ROS level plays a role in oxidative stress. Therefore, the body requires exogenous antioxidant supply to prevent the oxidative stress.(3)

The dietary antioxidant can be obtained naturally from plants or manufactured synthetically, but most of the natural antioxidants have better antioxidant activity compared with the synthetic one and are considerably safer.(4,5) Various compounds which have antioxidant activity have been isolated from plants, many of them are polyphenols including phenols, phenolic acids, flavonoids, tannins, and lignans.(1)

Phyllanthus niruri (*P. niruri*) is one of the herbal medicinal plants that have a broad range of properties, which are antiviral activities against hepatitis B, antimicrobial, hepatoprotective, anticancer, and hypocalcemic agent.(6) Several active phytochemicals have been discovered in *P. niruri*, such as flavonoids, alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins, and saponins.(7) A number of flavonoids had been successfully identified from *P. niruri*, including rutin and quercetin, which are well known to have significant antioxidants and chelating properties.(8-10) In the present study, antioxidant potential of *P. niruri* was investigated through 2,2'-azinobis-3-ethylbenzo-thiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazil (DPPH) and ferric reducing antioxidant power (FRAP) activity assays and also qualitative phytochemical screening assay with two flavonoid compounds rutin and quercetin for comparison. Protective antioxidant by plant products properties such as *P. niruri* may contribute as therapeutic drugs for free radical induced pathologies.

Methods

Preparation of *P. niruri* Extract

The stem and leaves of *P. niruri* were collected from Cianjur, West Java, Indonesia in February 2015. The plants were identified by herbarium staff, Department of Biology, School of Life Science and Technology, Bandung Institute of Technology, Bandung, Indonesia. The stem and leaves of *P. niruri* were mashed, extracted using ethanol 70% by a maceration method. The ethanol filtrate was filtered every 24 hours and collected until it was colorless. Evaporation was conducted to get concentrated ethanol extract in paste form. The extract was stored at -20˚C.(11-14)

Qualitative Phytochemical Screening Assay

The phytochemical assay was conducted on *P. niruri* extract using modified Fransworth method to qualitatively identified the presence of phytochemicals.(12,15-17) Phenol identification: the presence of phenol indicated by green/red/purple/blue or black color. Steroid/triterpenoid identification: the formation of green/blue color indicates the presence of steroid while red/orange sediment indicates the presence of triterpenoid. Saponin identification: saponin content was indicated by persistence of froth on the surface. Tannin identification: purple color formation indicates positive reaction for tannin. Terpenoid identification: terpenoid was indicated by the formation of purple color in the mixture. Flavonoid identification: the presence of flavonoid was shown by the formation of red or orange color. Alkaloid identification: the presence of yellow color indicated the positive result or presence alkaloid.

Total Phenol Content Assay

Total phenol content was determined using Folin-Ciocalteu reagent.(18) First, 15 µL samples were introduced into a 96-well microplate. Subsequently, 75 µL of Folin-Ciocalteu reagent (10%) and 60 µL of sodium carbonate (7.5%) were added. The plate was shaken and incubated at 50˚C for 10 mins. Finally, absorbance value was measured using a microplate reader at the wavelengths of 760 nm. The total phenol content was expressed as Gallic Acid equivalent (GAE), Quercetin equivalent (QE), and Rutin equivalent (RE) using the following formula:

$C = (c \times V) / m$

C: total content of phenolic compounds (µg/mg *P. niruri* extract in GAE, QE, or RE);
c: concentration of GAE, QE, or RE calculated from the regression equation (µg/mL);
V: volume of extract (mL);
m: weight of *P. niruri* extract (mg)

DPPH Assay

Briefly, 50 µL of samples were added to each well in a 96-well microplate. Then it was followed by adding 200 µL of DPPH (D9132, Sigma-Aldrich, St. Louis, MO, USA) solution (0.077 mmol/L in methanol) into the well. The mixture then was incubated in the dark for 30 mins at room temperature. Afterward, the absorbance was read using a microplate reader at 517 nm.(11,12,19,20) The radical scavenging activity was measured with following formula:

$\text{Scavenging \%} = (Ac - As) / Ac \times 100$

Ac: negative control absorbance (without sample)
As: sample absorbance

ABTS-reducing Activity Assay

ABTS solution was produced by reacting 14 mM ABTS (A1888, Sigma-Aldrich, St. Louis, MO, USA) and 4.9 mM calcium persulfate (1:1 volume ratio) for 16 hours in dark condition at room temperature, then the mixture was diluted with phosphate-buffered saline (PBS) (pH 7.4) until the absorbance of the solution was 0.70 ± 0.02 at wavelengths 745 nm. In brief, 2 µL of samples were added to each well at 96-well microplate, then fresh 198 µL ABTS solution were added. The absorbance was measured at 745 nm after the plate had been incubated for 6 min at 30˚C. The percentage

inhibition of ABTS radical (%) was determined by the ratio of reducing of ABTS absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control).(12,18) The median inhibitory concentration (IC_{50}) were also calculated.

FRAP Assay

The FRAP reagent was prepared freshly by mixing 10 mL of 300 mM acetate buffer (pH 3.6 adjusted with the addition of acetic acid) and 1 mL of 20 mM ferric chloride hexahydrate (Merck 1.03943.0250), and 1 mL of 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) (T1253, Sigma-Aldrich, St. Louis, MO, USA). In 96-well microplate, 7.5 µL of samples were mixed with 142.5 µL FRAP reagent then incubated for 30 min at 37˚C. The absorbance value was measured at 593 nm with a microplate reader (Thermo Scientific Multiscan GO, Thermo Fisher Scientific, Ratastie, Finland).(12,18,19)

Statistical Analysis

Each experiment was conducted in a triplicate manner. Data were expressed as a mean ± standard deviation. The differences between groups were statistically analyzed using one-way analysis of variance (one way ANOVA) with SPSS Statistics version 17.0 software. The probability values of $p<0.05$ were considered as statistically significant using Duncan's post hoc test.

Results

Phytochemical Screening of *P. niruri* Extract

The qualitative phytochemical screening assay on *P. niruri* extract showed that the extract had a high content of saponins (+++), a moderate content of phenols, flavonoids, and tannins (++). It also had a low content of steroids, but triterpenoids, terpenoids, and alkaloids were not detected in the screening (-) (Table 1).

Total Phenol Content (TPC)

The total phenolic content of *P. niruri* extract was measured using GAE, QE and RE as standard. Total phenol content of *P. niruri* extracts has value 61.36 ± 0.42 µg GAE/mg extract, 54.72 ± 0.39 µg QE/mg extract) and 424.29 ± 2.96 µg RE/mg extract. Those compounds were used since both are phenolic compounds that are known to be found in *P. niruri*.(7,17) The result revealed that total phenol content of *P. niruri* extract was high, and the highest value was achieved by rutin as standard.

Table 1. Qualitative phytochemical screening results of *P. niruri* extract.

Phytochemical content	*P. niruri* extract
Phenols	++
Steroids/ Triterpenoids	+/-
Terpenoids	-
Saponins	+++
Flavonoids	++
Tannins	++
Alkaloids	-

++++ (very high content); +++ (high content); ++ (moderate content); + (less content); - (not detected)

DPPH Scavenging Activity

The scavenging activity was increased along with the increase of the concentration of sample used. It can be seen at the highest concentration among samples, quercetin exhibited the highest DPPH scavenging activity, followed by *P. niruri* extract and the lowest was rutin (Table 2).

Quercetin had the lowest IC_{50} value followed by *P. niruri* extract then rutin (Table 3). The low IC_{50} suggested a high antioxidant activity, therefore quercetin has the most effective antioxidant activity in this DPPH assay.

ABTS-reducing Activity

Among the samples, it can be seen that both *P. niruri* extract and quercetin possess great ABTS-reducing activity, with the highest value in *P. niruri* extract and quercetin were similar and statistically not significant. Rutin had the lowest ABTS-reducing activities among three samples (Table 4). The value of IC_{50} of *P. niruri* extract, rutin, and quercetin in reducing the ABTS free radical in Table 5 revealed that *P. niruri* extract have low value of IC_{50}, even though the value was still slightly higher than quercetin. This assured that *P. niruri* extract exhibited effective antioxidant activity but still behind quercetin. Rutin was seen to have high IC_{50}, indicating this compound was not effective in reducing the ABTS radical.

FRAP Activity

The FRAP activity in this study showed that the activity was increased significantly in a concentration-dependent manner (Table 6). Both *P. niruri* and quercetin expressed high FRAP activity which indicates great antioxidant capability, while rutin remained to show the lowest activity. At low concentration, quercetin showed higher FRAP activity than *P. niruri*, but at the highest concentration, FRAP activity of *P. niruri* was comparable to quercetin.

Table 2. DPPH scavenging activity of *P. niruri* extract, rutin, and quercetin.

Concentration (µg/ml)	DPPH Scavenging Activity (%)		
	***P. niruri* extract**	**Rutin**	**Quercetin**
0.078	17.94 ± 0.07^{aB}	12.85 ± 0.22^{aA}	20.96 ± 0.54^{aC}
0.156	18.37 ± 0.14^{aB}	15.05 ± 0.32^{bA}	25.20 ± 0.87^{bC}
0.313	18.74 ± 0.40^{aB}	16.85 ± 0.18^{cA}	37.59 ± 0.58^{cC}
0.625	22.64 ± 2.29^{bB}	17.60 ± 0.17^{cA}	59.43 ± 0.37^{dC}
1.25	30.96 ± 0.12^{cB}	20.78 ± 0.35^{dA}	87.54 ± 0.06^{eC}
2.5	39.41 ± 0.87^{dB}	32.83 ± 0.82^{eA}	88.01 ± 0.35^{efC}
5	65.72 ± 2.34^{eB}	1.86 ± 0.69^{fA}	88.77 ± 0.45^{fgC}
10	85.54 ± 0.34^{fB}	75.40 ± 0.33^{gA}	89.16 ± 0.80^{gC}

*The data was presented as mean ± standard deviation. Different superscript small letters in the same column (among concentrations of the samples) and capital letters in the same row (among samples in various concentration) indicate significance at $p < 0.05$ (Duncan post hoc test).

Table 3. IC_{50} DPPH scavenging activity of *P. niruri* extract, rutin, and quercetin.

Samples	Linear equation	R^2	IC_{50} (µg/ml)	Average IC_{50} (µg/ml)
***P. niruri* extract**	y = 7.1691x + 19.562	0.96	4.25	4.24 ± 0.02
	y = 7.0874x + 19.884	0.96	4.25	
	y = 7.3017x + 19.203	0.95	4.22	
Rutin	y = 6.4588x + 14.465	0.98	5.5	5.56 ± 0.05
	y = 6.3406x + 14.532	0.99	5.59	
	y = 6.3814x + 14.442	0.99	5.57	
Quercetin	y = 57.638x + 18.201	0.98	0.55	0.55 ± 0.00
	y = 57.3x + 18.453	0.99	0.55	
	y = 57.274x + 18.365	0.99	0.55	

*The data was presented as mean ± standard deviation of IC_{50}, The most active DPPH scavenging activity was quercetin with the lowest value of IC_{50}

Table 4. ABTS-reducing activity of *P. niruri* extract, rutin, and quercetin.

Concentration (µg/ml)	ABTS-reducing Activity (%)		
	***P. niruri* extract**	**Rutin**	**Quercetin**
0.16	5.31 ± 0.49^{aB}	-0.30 ± 0.11^{aA}	7.72 ± 1.55^{aC}
0.31	13.17 ± 0.56^{bB}	$0.33 \pm 0.18a^{bA}$	17.86 ± 0.89^{bC}
0.63	26.06 ± 1.16^{cB}	0.78 ± 0.06^{bA}	32.59 ± 2.13^{cC}
1.25	45.56 ± 1.45^{dB}	1.81 ± 0.26^{cA}	60.60 ± 1.22^{dC}
2.5	76.18 ± 2.06^{eB}	7.94 ± 0.66^{dA}	94.93 ± 0.71^{eC}
5	98.76 ± 0.22^{fB}	12.76 ± 0.60^{eA}	99.55 ± 0.12^{fC}
10	99.10 ± 0.29^{fB}	29.08 ± 0.65^{fA}	99.55 ± 0.24^{fB}

*The data was presented as mean ± standard deviation. Different superscript small letters in the same column (among concentrations of the samples) and capital letters in the same row (among samples in various concentration) indicate significance at $p < 0.05$ (Duncan post hoc test).

Table 5. IC_{50} ABTS-reducing activity of *P. niruri* extract, rutin, and quercetin.

Samples	Linear equation	R^2	IC_{50} (µg/ml)	Average IC_{50} (µg/ml)
***P. niruri* extract**	y = 30.224x + 4.7132	0.99	1.5	1.53 ± 0.03
	y = 28.534x + 5.566	0.98	1.56	
	y = 30.053x + 3.4504	0.99	1.55	
Rutin	y = 2.9161x - 0.8221	0.99	17.43	17.16 ± 0.23
	y = 2.996x - 1.1215	0.99	17.06	
	y = 2.9908x - 0.8332	0.99	17	
Quercetin	y = 36.897x + 6.4942	0.98	1.18	1.17 ± 0.02
	y = 36.979x + 7.6461	0.97	1.15	
	y = 36.131x + 7.5053	0.98	1.18	

*The data was presented as mean ± standard deviation of IC_{50}, The most active DPPH scavenging activity was quercetin with the lowest value of IC_{50}

Table 6. FRAP activity of P. niruri extract, rutin, and quercetin.

Concentration (µg/ml)	FRAP activity (µM Fe(II)/ µg extract)		
	***P. niruri* extract**	**Rutin**	**Quercetin**
0.78	72.58 ± 0.83^{aB}	18.95 ± 1.82^{aA}	116.79 ± 6.90^{aC}
1.56	123.92 ± 0.40^{bB}	23.65 ± 0.39^{bA}	211.50 ± 1.73^{bC}
3.13	213.44 ± 1.78^{cB}	31.85 ± 0.46^{cA}	316.99 ± 5.28^{cC}
6.25	303.53 ± 1.79^{dB}	53.19 ± 2.20^{dA}	348.86 ± 4.73^{dC}
12.5	358.49 ± 3.16^{eB}	100.58 ± 1.46^{eA}	369.11 ± 5.28^{eC}
25	373.95 ± 2.87^{fB}	189.13 ± 2.75^{fA}	372.28 ± 0.32^{eB}

*The data was presented as mean ± standard deviation. Different superscript small letters in the same column (among concentrations of the samples) and capital letters in the same row (among samples in various concentration) indicate significance at $p < 0.05$ (Duncan post hoc test).

Discussion

The qualitative phytochemical screening of *P. niruri* extract showed the presence of high content of saponin, the moderate content of phenol, flavonoid, tannin and low content of steroid. However, triterpenoid, terpenoid, and alkaloid were undetected in the extract with the method used in this study (Table 1). Based on other studies, *P. niruri* contains tannins, terpenes, flavonoids, alkaloids, and saponins found in the leaves, stem, and roots of the plant.(7) Calixto, *et al.,* reported that *P. niruri* contained flavonoid quercetin, quercitrin, isoquercitrin, astragalin, rutin and physetinglucoside.(21) Geraniin, repandusinic acid and corilagin are tannins found in *P. niruri*.(7) The alkaloid isolated from *P. niruri* is a securinega-type alkaloid, epibubbialine.(22) The free radical scavenging activity of this plant could be due to the presence of various bioactive compounds such as alkaloids, flavonoids, and polyphenols has antioxidant capabilities.(23)

The antioxidant assay of *P. niruri* extract, rutin and quercetin revealed that quercetin possessed the greatest antioxidant activity, followed closely by *P. niruri* extract in DPPH and ABTS assay (Table 2, 3). In FRAP assay, *P. niruri* has the highest activity at the concentration of 25 µg/mL, that comparable to quercetin. The differences of antioxidant value among assays indicate that each assay determines different aspects in measuring antioxidant capacity and distinct radicals and mechanisms of reaction occurred. (24) Rutin exhibited the lowest activity among the samples in DPPH (Table 2, 3), ABTS (Table 4, 5), and FRAP assay (Table 6) based on the result, suggesting that rutin has the least antioxidant potential.

The high antioxidant activity of *P. niruri* might be related to the numerous active compounds in the plant, including the phytochemicals flavonoids and

polyphenols.(25) This was in accordance with the current phytochemical screening results, which detected different phytochemical contents. Based on the total phenol content assay in this study, it was also revealed that *P. niruri* contained high phenol content and the highest total phenol content was obtained using rutin as equivalent, with the value of TPC was 424.29 µg RE/mg extract. The OH groups in phenols related to antioxidant activity, that can reduce of amount oxidation by transferring an H atom to chain-carrying radicals.(26)

Mediani, *et al.,* reported that *P. niruri* contained a high amount of rutin, followed by gallic acid, quercetin.(27) The phenolic compound gallic acid and ellagic acid found in *P. niruri* have been demonstrated to possess antioxidant activity and cancer chemopreventive abilities.(28) Rutin can reduce the risk of arteriosclerosis, anti-inflammatory and vasoactive properties, reduce coronary heart disease, and anticancer properties.(29, 30) In another study, quercetin has scavenging radicals activity and chelating transition metal ions that play a role in preventing oxidation of low-density lipoproteins (LDL) to against cancer, atherosclerosis, and inflammation.(31-33)

In the present study, rutin has the lower antioxidant activity than quercetin. These were consistent with Ahmeda, *et al.,* who reported that quercetin has higher antioxidant activity than rutin in the DPPH assay due to the steric hindrance in rutin.(34,35) In another study conducted by Firuzi, *et al.,* it was also demonstrated that quercetin had significantly higher antioxidant activity than rutin in FRAP assay, with FRAP value after 4 min of quercetin and rutin were 65.0 and 21.9 µM, respectively.(36)

Those results were assumed to be caused by the absence of 2,3-double bond or 3-OH in the C ring in the rutin structure, which were presumably important in determining antioxidant activity.(36) Among polyphenolic compounds, several factors could be responsible for increasing radical scavenging effectiveness. Those factors are including ortho-dihydroxy structure in the B ring, 2,3-double bond in conjugation with a 4-oxo function in the C-ring, hydroxy groups in positions 3 and 5 in the A ring, or the angle between the rings in the compound structure.(37-40)

Conclusion

In summary, *P. niruri* extract and quercetin have great potential as a natural antioxidant source.

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