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Phytochemical Screening and Antioxidant Assay of *Phyllantus niruri* Extract and Flavonoid Compounds Rutin, Quercetin

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Running Title: Antioxidant of *Phyllantus niruri* Extract and Compounds

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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 antioxidant to protect against free radicals and plants are a good source of natural antioxidants. *Phyllanthus niruri* has been known to possess several medicinal properties and contain numerous active phytochemical, thus in this research we conducted phytochemical screening and antioxidant assay of *P. niruri* extract along with the compounds rutin and quercetin, which are flavonoids that also possess medicinal properties. **OBJECTIVE:** This study was conducted to determine *Phyllantus niruri*, rutin and quercetin have antioxidant activity. **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 measured total phenolic content, 2,2-diphenyl-1-picrylhydrazil (DPPH), 2,2'-

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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 contains phytochemical bioactive compounds and along with quercetin, both have great potential as a natural antioxidant source.
KEYWORDS: Antioxidant, phytochemical, *Phyllanthus niruri*, quercetin, rutin, free radical

INTRODUCTION

Reactive Oxygen Species (ROS) may damage the body itself by attacking lipids in 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) However, excessive ROS level still could play 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 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)

P. niruri is one of the herbal medicinal plants that have a wide 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 successfully identified from *P. niruri*, including rutin and quercetin, it well known that have significant antioxidants and chelating properties.(6,10,11) In this study, we investigated the phytochemical content of *P. niruri* qualitatively as well as antioxidant activity of *P. niruri*, along with two flavonoid compounds rutin and quercetin for comparison.

MATERIALS AND METHODS:

Preparation of *Phyllanthus niruri* Extract:

The stem and leaves of *P. niruri* were collected from Cianjur district, 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, West Java, Indonesia. The stem and leaves of *P. niruri* were mashed, extracted using ethanol 70% by a maceration method. Every 24 h the ethanol filtrate was filtered and collected until it was colourless. Evaporation was conducted to get concentrated ethanol extract in paste form. The extract was stored at -20 ^{o}C.(12)

Qualitative Phytochemical Screening Assay:

The phytochemical assay was conducted on *P. niruri* extract using modified Fransworth method to identified qualitatively presence of phytochemicals.(13,14)

Phenol identification

The colour formation of green/red/purple/blue/black shows presence of phenol.

Steroid / triterpenoid identification

The formation of green/blue colour 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 colour formation indicates positive reaction for tannin.

Terpenoid identification

Terpenoid was indicated by the formation of purple colour on the mixture.

Flavonoid identification

The presence of flavonoid was shown by the formation of red or orange colour.

Alkaloid identification

The presence of yellow colour indicated the positive result or presence alkaloid.

Total Phenol Content Assay:

Total phenol content was determined using Folin-Ciocalteu reagent according to Widowati *et al.* method. (15) First, 15 µl samples were introduced into a 96-well microplate. Subsequently, 75 µl of Folin-

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Ciocalteu reagent (10%) and 60 µl of sodium carbonate (7.5%) were added. The plate was shaken and incubated at 50 °C for 10 min. Finally, absorbance value was measured using a microplate reader at the wavelength 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)

2,2-Diphenyl-1-picrylhydrazil (DPPH) assay:

Briefly, 50 µl of samples were added to each well in a 96-well microplate. It was then followed by addition of 200 µl of 2,2-Diphenyl-1-picrylhydrazil (DPPH) (Sigma-Aldrich) solution (0.077 mmol/l in methanol) into the well. The mixture then incubated in the dark for 30 min at room temperature. Afterwards, the absorbance was read using a microplate reader at 517 nm.(12) The radical scavenging activity was measured using the following formula:

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

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

ABTS-reducing activity assay:

$ABTS^{\bullet+}$ solution was produced by reacting 14 mM ABTS and 4.9 mM calcium persulfate (1:1 volume ratio) for 16 h in dark condition at room temperature, then the mixture was diluted with PBS (pH 7.4) until the absorbance of the solution was 0.70 ± 0.02 at wavelength 745 nm. In brief, 2 µl of samples were added to each well at 96-well microplate, then to the samples the fresh 198 µl $ABTS^{\bullet+}$ solution were added. The absorbance was measured at 745 nm after the plate incubated for 6 min at 30 °C. The percentage inhibition of ABTS radical (%) was determined by the ratio of reducing of $ABTS^{\bullet+}$ absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control). The median inhibitory concentration (IC_{50}) were also calculated.

Ferric reducing antioxidant power (FRAP) assay:

The FRAP reagent was prepared freshly by mixing 10 ml of acetate buffer 300 mM (pH 3.6 adjusted with addition of acetic acid), 1 ml of ferric chloride hexahydrate 20 mM dissolved in distilled water, and 1 ml of 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) 10 mM dissolved in HCl 40 mM [24]. 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 (15,16).

Statistical analysis:

Each experiment was conducted in a triplicate manner. Data were expressed as mean ± standard deviation. The differences between groups were statistically analysed using one-way analysis of variance (ANOVA) with SPSS Statistics version 17.0 software. 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)

Total phenol content of *P. niruri* extract was shown on Table 2. The total phenolic content of *P. niruri* extract was measured using gallic acid (GAE), quercetin (QE), and rutin (RE) as standard. Those compounds were used since both are phenolic compounds that known can be found in *P.niruri*.(7) The result revealed that total phenol content of *P. niruri* extract was high, and the highest value was achieved using rutin as standard (Table 2).

2,2-Diphenyl-1-picrylhydrazil (DPPH) Scavenging Activity

The scavenging activity was increased along with the increase of the concentration of sample used, suggesting the activity was concentration-dependent. It can be seen at the highest concentration among samples, quercetin exhibited the highest DPPH scavenging activity, followed by *Phyllanthus niruri* extract and the lowest was rutin (Table 3).

Quercetin had the lowest IC_{50} value followed by *P. niruri* extract then rutin (Table 4). The low IC_{50} suggested high antioxidant activity, therefore based on the IC_{50} value quercetin has the greatest and 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 5). The value of IC_{50} of *P. niruri* extract, rutin, and quercetin in reducing the ABTS free radical in Table 6 revealed that *P. niruri* extract have low value of IC_{50}, even though the value was still slightly higher than quercetin. This reassured 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.

Ferric reducing antioxidant power (FRAP) activity

The FRAP activity in this study showed that the activity was increased significantly in a concentration-dependent manner (Table 7). *P. niruri* and quercetin were both expressed high FRAP activity, indicating both have great antioxidant capability whilst rutin remained to showed lower activity compared to the two. 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.

DISCUSSION

The qualitative phytochemical screening of *P. niruri* extract showed the presence of high content of saponin, along with 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* contain tanins, 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.(17) Geraniin, repandusinic acid, and corilagin are tannins found in *P. niruri*.(7) The alkaloid that has been isolated from *P. niruri* is a securinega-type alkaloid, epibubbialine.(18)

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 3,4). In FRAP assay, *Phyllanthus niruri* have the highest activity at concentration of 25 µg/ml, that comparable to quercetin. The differences of antioxidant value among assays used indicate that each assay determines different aspects in measuring antioxidant capacity and distinct radicals and mechanisms of reaction occurred.(19) Rutin exhibited the lowest activity among the samples in DPPH, ABTS, and FRAP assay based on the result (Table 3-5), suggesting that rutin has the least antioxidant potential.

The high antioxidant activity of *P. niruri* said to be might related to the numerous active compounds in the plant, including the phytochemicals flavonoids and polyphenols.(20) These was in accordance with the current phytochemical screening results, which detected various 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 (Table 2). The antioxidant activity of phenols are related to their OH groups, as they can reduce the rates of oxidation of organic matter by transferring an H atom (from OH groups) to the chain-carrying ROO* radicals.(21) Mediani *et al.* reported that *P. niruri* contained a high amount of rutin, followed by gallic acid, quercetin.(22) The phenolic compound gallic acid and ellagic acid found in *P. niruri* have been demonstrated to possessed antioxidant activity and cancer chemopreventive abilities.(23) Rutin is known for its anti-inflammatory and vasoactive properties, reduce the risk of arteriosclerosis, reduce coronary heart disease through diminishing of platelet aggregation, and a protective agent against carcinogenesis.(24,25) Quercetin has reported to have capability in preventing oxidation of low-density lipoproteins (LDL) by scavenging free radicals and chelating transition metal ions (26), thus may aid in the prevention of certain disease including cancer, atherosclerosis, and chronic inflammation.(27,28)

Flavonoid compound quercetin in the present study revealed to exhibit the highest antioxidant activity, whilst rutin had the lowest. These was consistent with Ahmeda *et al.* study, reported that quercetin has higher antioxidant activity than rutin in the DPPH assay which might due to the steric hindrance in rutin.(29,30) 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.(31) Those result were stated might due to the absence of 2,3-double bond or 3-OH in the C ring in the rutin structure, as in FRAP assay those factors presumably important in determining antioxidant activity.(31) Among polyphenolic compounds, several factors could be responsible for increasing radical scavenging effectiveness, such as an 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, and the angle between the rings in the compound structure.(32,33-35)

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ACKNOWLEDGEMENT

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Dwi Davidson Rihibiha, Hayatun Nufus, Bejo Ropii, Putri Ramadhany from Biomolecular and Biomedical Research Center, Aretha Medika Utama, Bandung, West Java, Indonesia for their valuable assistance.

CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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Tables

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- Each table has to be mentioned/discussed in the paragraph.
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Table 1: Qualitative phytochemical screening results of *Phyllanthus 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)

Table 2: Total phenol content of *P. niruri* extract

Sample	Total Phenol Content		
	(µg GAE/mg extract)	(µg QE/mg extract)	(µg RE/mg extract)
P. niruri extract	61.36 ± 0.42	54.72 ± 0.39	424.29 ± 2.96

*The data was presented as mean ± standard deviation.

Table 3: 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 and capital letters in the same row indicate significance at P<0.05 (Duncan post hoc test).

Table 4: 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	
	y = 7.0874x + 19.884	0.96	4.25	4.24 ± 0.02
	y = 7.3017x + 19.203	0.95	4.22	
Rutin	y = 6.4588x + 14.465	0.98	5.50	
	y = 6.3406x + 14.532	0.99	5.59	5.56 ± 0.05
	y = 6.3814x + 14.442	0.99	5.57	
Quercetin	y = 57.638x + 18.201	0.98	0.55	
	y = 57.3x + 18.453	0.99	0.55	0.55 ± 0.00
	y = 57.274x + 18.365	0.99	0.55	

*The data was presented as mean ± standard deviation.

Table 5: 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 ± 0.18^{abA}	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 and capital letters in the same row indicate significance at P<0.05 (Duncan post hoc test).

Table 6: 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	1.50	
	y = 28.534x + 5.566	0.98	1.56	1.53 ± 0.03
	y = 30.053x + 3.4504	0.99	1.55	
Rutin	y = 2.9161x - 0.8221	0.99	17.43	
	y = 2.996x - 1.1215	0.99	17.06	17.16 ± 0.23
	y = 2.9908x - 0.8332	0.99	17.00	
Quercetin	y = 36.897x + 6.4942	0.98	1.18	
	y = 36.979x + 7.6461	0.97	1.15	1.17 ± 0.02
	y = 36.131x + 7.5053	0.98	1.18	

*The data was presented as mean ± standard deviation.

Table 7: 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}
12.50	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 and capital letters in the same row indicate significance at P<0.05 (Duncan post hoc test).

Phytochemical Screening and Antioxidant Assay of *Phyllantus Niruri* Extract and Flavonoid Compounds Rutin, Quercetin

ABSTRACT

BACKGROUND: Normal metabolism of oxygen and exogenous factors constantly generate free radicals which could be harmful to the human body. Human need antioxidant to provide protection against free radicals, thus ~~and~~ plants are ~~a~~ good source of natural antioxidants. *Phyllanthus niruri* has been known to possess several medicinal properties and contain numerous active phytochemical, thus in this research we conducted phytochemical screening and antioxidant assay of *P. niruri* extract along with the compounds rutin and quercetin, which are flavonoids ~~that also~~ 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 ~~measured~~ 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 contains phytochemical bioactive compounds and along with quercetin, both have great potential as ~~a~~ natural antioxidant source.

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

INTRODUCTION

Reactive Oxygen Species (ROS) lead~~sing~~ to damaginge 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 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)

P. niruri is one of the herbal medicinal plants that have a wide 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,

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tannins, coumarins, and saponins.(7) A number of flavonoids had been successfully identified from *P. niruri*, including rutin and quercetin, ~~it~~ that are well known ~~that~~ to have significant antioxidants and chelating properties.(8,9,10) ~~In this study, we investigated~~This research investigated the phytochemical content of *P. niruri* qualitatively as well as antioxidant activity of *P. niruri*, along with two flavonoid compounds rutin and quercetin for comparison.

MATERIALS AND METHODS:

Preparation of *P. niruri* Extract:

The stem and leaves of *P. niruri* were collected from Cianjur district, 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, West Java, Indonesia. The stem and leaves of *P. niruri* were mashed, extracted using ethanol 70% by a maceration method. Every 24 h the ethanol filtrate was filtered and collected until it was colourless. Evaporation was conducted to get concentrated ethanol extract in paste form. The extract was stored at -20 °C.(11)

Qualitative Phytochemical Screening Assay:

The phytochemical assay was conducted on *P. niruri* extract using modified Fransworth method to identified qualitatively presence of phytochemicals.(12,13,14,15)

Phenol identification

The presence of phenol indicated by colour green/red/purple/blue, or black.

Steroid / triterpenoid identification

The formation of green/blue colour 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 colour formation indicates positive reaction for tannin.

Terpenoid identification

Terpenoid was indicated by the formation of purple colour on the mixture.

Flavonoid identification

The presence of flavonoid was shown by the formation of red or orange colour.

Alkaloid identification

The presence of yellow colour indicated the positive result or presence alkaloid.

Total Phenol Content Assay:

Total phenol content was determined using Folin-Ciocalteu reagent ~~according to Widowati *et al*. method.~~(16) 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)

2,2-Diphenyl-1-picrylhydrazil (DPPH) assay:

Briefly, 50 µl of samples were added to each well in a 96-well microplate. ~~It was then~~Then it was followed by ~~addition~~ adding ~~of~~ 200 µl of 2,2-Diphenyl-1-picrylhydrazil (DPPH) (Sigma-Aldrich) solution (0.077 mmol/l in methanol) into the well. The mixture then was incubated in the dark for 30 mins at room temperature. Afterwards, the absorbance was read using a microplate reader at 517 nm.(11,17) The radical scavenging activity was measured using the following formula:

Scavenging % = (Ac – As) / Ac x 100
Ac: negative control absorbance (without sample)
As: sample absorbance

ABTS-reducing activity assay:

$ABTS^{\bullet+}$ solution was produced by reacting 14 mM ABTS and 4.9 mM calcium persulfate (1:1 volume ratio) for 16 h in dark condition at room temperature, then the mixture was diluted with 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 ~~to the samples the~~ fresh 198 µl $ABTS^{\bullet+}$ 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 $ABTS^{\bullet+}$ absorbance in the presence of the sample relative to the absorbance in the absence of the sample (negative control).(14,15) The median inhibitory concentration (IC_{50}) were also calculated.

Ferric reducing antioxidant power (FRAP) assay:

The FRAP reagent was prepared freshly by mixing 10 ml of acetate buffer 300 mM (pH 3.6 adjusted with addition of acetic acid), 1 ml of ferric chloride hexahydrate 20 mM dissolved in distilled water, and 1 ml of 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) 10 mM dissolved in HCl 40 mM. 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.(15,16,17)

Statistical analysis:

Each experiment was conducted in a triplicate manner. Data were expressed as mean ± standard deviation. The differences between groups were statistically analysed using one-way analysis of variance (ANOVA) with SPSS Statistics version 17.0 software. 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 gallic acid (GAE), quercetin (QE), and rutin (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 ~~can be~~to be found in *P.niruri*.(7,14) The result revealed that total phenol content of *P. niruri* extract was high, and the highest value was achieved using rutin as standard.

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2,2-Diphenyl-1-picrylhydrazil (DPPH) Scavenging Activity

The scavenging activity was increased along with the increase of the concentration of sample used, suggesting the activity was concentration-dependent. 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 high antioxidant activity, therefore based on the IC_{50} value quercetin has the greatest and 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 reassured 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.

Ferric reducing antioxidant power (FRAP) activity

The FRAP activity in this study showed that the activity was increased significantly in a concentration-dependent manner (Table 6). *P. niruri* and quercetin were both expressed high FRAP activity, indicating both have great antioxidant capability whilst rutin remained to showed lower activity compared to the two. 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.

DISCUSSION

The qualitative phytochemical screening of *P. niruri* extract showed the presence of high content of saponin, along with 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* contain tanins, 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.(18) Geraniin, repandusinic acid, and corilagin are tannins found in *P. niruri*.(7) The alkaloid that has been isolated from *P. niruri* is a securinega-type alkaloid, epibubbialine.(19)

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 and Table 3). In FRAP assay, *P. niruri* have the highest activity at concentration of 25 µg/ml, that comparable to quercetin. The differences of antioxidant value among assays used indicate that each assay determines different aspects in measuring antioxidant capacity and distinct radicals and mechanisms of reaction occurred.(20) Rutin exhibited the lowest activity among the samples in DPPH (Table 2 and Table 3), ABTS (Table 4 and Table 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* said to be might related to the numerous active compounds in the plant, including the phytochemicals flavonoids and polyphenols.(21) These was in accordance with the current phytochemical screening results, which detected various 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.(22) Mediani *et al.* reported that *P. niruri* contained a high amount of rutin, followed by gallic acid, quercetin.(23) The phenolic compound gallic acid and ellagic acid found in *P. niruri* have been demonstrated to possessed antioxidant activity and cancer chemopreventive abilities.(24) Rutin can reduce the risk of arteriosclerosis,

anti-inflammatory and vasoactive properties, reduce coronary heart disease, and anticancer properties.(25,26) In other study, quercetin has scavenging radicals activity and chelating transition metal ions that has play role in preventing oxidation of low-density lipoproteins (LDL) to against cancer, atherosclerosis, and inflammation.(27,28,29)

In the present study, rutin has the lower antioxidant activity than quercetin. These was consistent with Ahmeda *et al.* study, reported that quercetin has higher antioxidant activity than rutin in the DPPH assay which might due to the steric hindrance in rutin.(30,31) 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.(32) Those result were stated might due to the absence of 2,3-double bond or 3-OH in the C ring in the rutin structure, as in FRAP assay those factors presumably important in determining antioxidant activity.(32) Among polyphenolic compounds, several factors could be responsible for increasing radical scavenging effectiveness, such as an 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, and the angle between the rings in the compound structure.(33,34,35,36)

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CONFLICT OF INTEREST

All contributing authors declare no conflicts of interest.

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Tables

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)

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 and capital letters in the same row 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	
	y = 7.0874x + 19.884	0.96	4.25	4.24 ± 0.02
	y = 7.3017x + 19.203	0.95	4.22	
Rutin	y = 6.4588x + 14.465	0.98	5.50	
	y = 6.3406x + 14.532	0.99	5.59	5.56 ± 0.05
	y = 6.3814x + 14.442	0.99	5.57	
Quercetin	y = 57.638x + 18.201	0.98	0.55	
	y = 57.3x + 18.453	0.99	0.55	0.55 ± 0.00
	y = 57.274x + 18.365	0.99	0.55	

*The data was presented as mean ± standard deviation 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 ± 0.18^{abA}	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 and capital letters in the same row 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	1.50	
	y = 28.534x + 5.566	0.98	1.56	1.53 ± 0.03
	y = 30.053x + 3.4504	0.99	1.55	
Rutin	y = 2.9161x - 0.8221	0.99	17.43	
	y = 2.996x - 1.1215	0.99	17.06	17.16 ± 0.23
	y = 2.9908x - 0.8332	0.99	17.00	
Quercetin	y = 36.897x + 6.4942	0.98	1.18	
	y = 36.979x + 7.6461	0.97	1.15	1.17 ± 0.02
	y = 36.131x + 7.5053	0.98	1.18	

*The data was presented as mean ± standard deviation 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}
12.50	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 and capital letters in the same row indicate significance at $P<0.05$ (Duncan post hoc test).

Dear Dr. Wahyu Widowati,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "Antioxidant Activity of Phyllanthus niruri Extract, Rutin and Quercetin". Our decision is to: **Accept Submission**.

While waiting for the actual assignment of your manuscript, your manuscript is now available online in our Article In Press section. Congratulations on your interesting research, and thank you for allowing us to publish this valuable material.

Best regards,
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